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| <p>(21) International Application Number: PCT/US98/21232</p> <p>(22) International Filing Date: 9 October 1998 (09.10.98)</p> <p>(30) Priority Data: 60/061,676 10 October 1997 (10.10.97) US</p> <p>(71) Applicant: CYTOVIA, INC. [US/US]; 6650 Nancy Ridge Drive, San Diego, CA 92121 (US).</p> <p>(72) Inventors: KEANA, John, F., W.; 3854 Onyx Street, Eugene, OR 07405 (US). CAI, Sui, Xiong; 3633 Berryfield Court, San Diego, CA 92130 (US). GUASTELLA, John; 40 Exeter, Irvine, CA 92612 (US). YANG, Wu; 1 Corte Trovata, Irvine, CA 92606 (US). DREWE, John, A.; 2300 Fairview Road #G204, Costa Mesa, CA 92626 (US).</p> <p>(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C, Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).</p> | | <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p> |
| <p>(54) Title: DIPEPTIDE APOPTOSIS INHIBITORS AND THE USE THEREOF</p> <div style="text-align: center; margin: 20px 0;"> $\begin{array}{c} \text{CO}_2\text{R}_3 \\ \\ \text{R}_1\text{--AA--NH--CH--C--R}_2 \\ \qquad \qquad \qquad \\ \text{H} \qquad \qquad \qquad \text{O} \end{array} \quad (I)$ </div> <p>(57) Abstract</p> <p>The present invention is directed to novel dipeptides thereof, represented by general Formula (I) where R₁–R₃ and AA are defined herein. The present invention relates to the discovery that compounds having Formula (I) are potent inhibitors of apoptotic cell death. Therefore, the inhibitors of this invention can retard or block cell death in a variety of clinical conditions in which the loss of cells, tissues or entire organs occurs.</p> | | |

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Dipeptide Apoptosis Inhibitors and the Use Thereof

Background of the Invention

Field of the Invention

This invention is in the field of medicinal chemistry. In particular, the invention relates to dipeptides which are potent inhibitors of apoptosis. The invention also relates to the use of these dipeptides for reducing or treating apoptotic cell death.

Description of Background Art

Organisms eliminate unwanted cells by a process variously known as regulated cell death, programmed cell death or apoptosis. Such cell death occurs as a normal aspect of animal development as well as in tissue homeostasis and aging (Glucksmann, A., *Biol. Rev. Cambridge Philos. Soc.* 26:59-86 (1951); Glucksmann, A., *Archives de Biologie* 76:419-437 (1965); Ellis *et al.*, *Dev.* 112:591-603 (1991); Vaux *et al.*, *Cell* 76:777-779 (1994)). Apoptosis regulates cell number, facilitates morphogenesis, removes harmful or otherwise abnormal cells and eliminates cells that have already performed their function. Additionally, apoptosis occurs in response to various physiological stresses, such as hypoxia or ischemia (PCT published application WO96/20721).

There are a number of morphological changes shared by cells experiencing regulated cell death, including plasma and nuclear membrane blebbing, cell shrinkage (condensation of nucleoplasm and cytoplasm), organelle relocation and compaction, chromatin condensation and production of apoptotic bodies (membrane enclosed particles containing intracellular material) (Orrenius, S., *J. Internal Medicine* 237:529-536 (1995)).

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Apoptosis is achieved through an endogenous mechanism of cellular suicide (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34). A cell activates its internally encoded suicide program as a result of either internal or external signals. The suicide program is executed through the activation of a carefully regulated genetic program (Wyllie *et al.*, *Int. Rev. Cyt.* 68: 251 (1980); Ellis *et al.*, *Ann. Rev. Cell Bio.* 7: 663 (1991)). Apoptotic cells and bodies are usually recognized and cleared by neighboring cells or macrophages before lysis. Because of this clearance mechanism, inflammation is not induced despite the clearance of great numbers of cells (Orrenius, S., *J. Internal Medicine* 237:529-536 (1995)).

Mammalian interleukin-1 β (IL-1 β) plays an important role in various pathologic processes, including chronic and acute inflammation and autoimmune diseases (Oppenheim, J. H. et. al. *Immunology Today*, 7, 45-56 (1986)). IL-1 β is synthesized as a cell associated precursor polypeptide (pro-IL-1 β) that is unable to bind IL-1 receptors and is biologically inactive (Mosley *et al.*, *J. Biol. Chem.* 262:2941-2944 (1987)). By inhibiting conversion of precursor IL-1 β to mature IL-1 β , the activity of interleukin-1 can be inhibited. Interleukin-1 β converting enzyme (ICE) is a protease responsible for the activation of interleukin-1 β (IL-1 β) (Thornberry, N.A., *et al.*, *Nature* 356: 768 (1992); Yuan, J., *et al.*, *Cell* 75: 641 (1993)). ICE is a substrate-specific cysteine protease that cleaves the inactive prointerleukin-1 to produce the mature IL-1. The genes that encode for ICE and CPP32 are members of the mammalian ICE/Ced-3 family of genes which presently includes at least twelve members: ICE, CPP32/Yama/Apopain, mICE2, ICE4, ICH1, TX/ICH-2, MCH2, MCH3, MCH4, FLICE/MACH/MCH5, ICE-LAP6 and ICE_{rel}III. The proteolytic activity of this family of cysteine proteases, whose active site cysteine residue is essential for ICE-mediated apoptosis, appears critical in mediating cell death (Miura *et al.*, *Cell* 75: 653-660 (1993)).

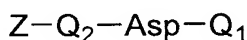
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This gene family has recently been named caspases (Alnemri, E. S. *et al. Cell*, 87:171 (1996)).

IL-1 is also a cytokine involved in mediating a wide range of biological responses including inflammation, septic shock, wound healing, hematopoiesis and growth of certain leukemias (Dinarello, C.A., *Blood* 77:1627-1652 (1991); diGiovine *et al., Immunology Today* 11:13 (1990)).

Many potent caspase inhibitors have been prepared based on the peptide substrate structures of caspases. However, in contrast to their potency *in vitro*, no inhibitors with good efficacy ($IC_{50} < 1 \mu M$) in whole-cell models of apoptosis have been reported (Thornberry, N. A. *Chem. Biol.* 5:R97-103 (1998)). Therefore the need exists for cell death inhibitors that show efficacy ($IC_{50} < 1 \mu M$) in whole-cell models of apoptosis and are active in animal models of apoptosis. These inhibitors thus can be employed as therapeutic agents to treat disease states in which regulated cell death and the cytokine activity of IL-1 play a role.

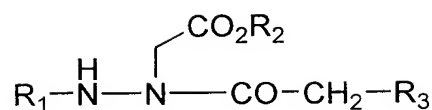
WO 93/05071 disclosed peptide ICE inhibitors with the formula:



wherein Z is an N-terminal protecting group; Q_2 is 0 to 4 amino acids such that the sequence Q_2-Asp corresponds to at least a portion of the sequence Ala-Tyr-Val-His-Asp; Q_1 comprises an electronegative leaving group. Exemplary dipeptides are Boc-His-Asp-CH₂F, Boc-Tyr-Asp-CH₂F, Boc-Phe-Asp-CH₂F, Ac-His-Asp-CH₂F, Ac-Tyr-Asp-CH₂F, Ac-Phe-Asp-CH₂F, Cbz-His-Asp-CH₂F, Cbz-Tyr-Asp-CH₂F and Cbz-Phe-Asp-CH₂F.

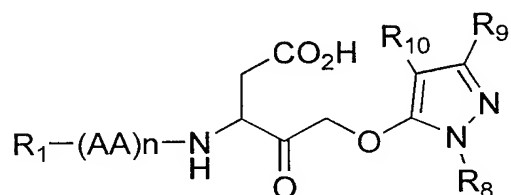
WO 96/03982 disclosed aspartic acid analogs as ICE inhibitors with the formula:

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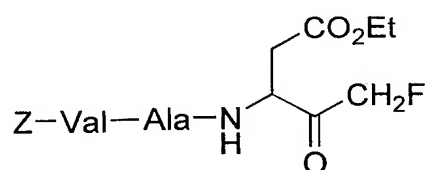
wherein R_2 is H or alkyl; R_3 is a leaving group such as halogen; R_1 is heteroaryl-CO or an amino acid residue.

US patent 5,585,357 disclosed peptidic ketones as ICE inhibitors with the formula:



wherein n is 0-2; each AA is independently L-valine or L-alanine; R_1 is selected from the group consisting of N-benzyloxycarbonyl and other groups; R_8 , R_9 , R_{10} are each independently hydrogen, low alkyl and other groups.

Revesz *et al.* (*Tetrahedron Lett.* 35, 9693-9696, **1994**) reported the preparation of ethyl ester tripeptide:

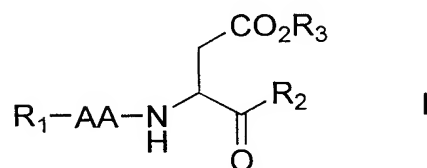


as a prodrug of the corresponding acid which is a potent ICE inhibitor.

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Summary of the Invention

The invention relates to dipeptides of formula I:



wherein R_1 is an N-terminal protecting group; AA is a residue of any natural α -amino acid, or β -amino acid; R_2 is H or CH_2R_4 where R_4 is an electronegative leaving group, and R_3 is alkyl or H, provided that AA is not His, Tyr, Pro or Phe.

The invention relates to the discovery that the dipeptide-based caspase inhibitors represented by Formula I, though less potent in enzyme assays than tri- and tetrapeptide inhibitors on the enzymes, are surprisingly potent inhibitors of apoptosis in cell based systems. These compounds are systemically active *in vivo* and are potent inhibitors of antiFas-induced lethality in a mouse liver apoptosis model and have robust neuroprotective effects in a rat model of ischemic stroke.

The invention also relates to the use of the dipeptides of the invention for reducing, preventing or treating maladies in which apoptotic cell death is either a causative factor or a result. Examples of uses for the present invention include protecting the nervous system following focal ischemia and global ischemia; treating neurodegenerative disorders such as Alzheimer's disease, Huntington's Disease, prion diseases, Parkinson's Disease, multiple sclerosis, amyotrophic lateral sclerosis, ataxia, telangiectasia, and spinobulbar atrophy; treating heart disease including myocardial infarction, congestive heart failure and cardiomyopathy; treating retinal disorders; treating autoimmune disorders including lupus erythematosus, rheumatoid arthritis, type I diabetes, Sjögren's

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syndrome and glomerulonephritis; treating polycystic kidney disease and anemia/erythropoiesis; treating immune system disorders, including AIDS and SCIDS; reducing or preventing cell, tissue and organ damage during transplantation; reducing or preventing cell line death in industrial biotechnology; reducing or preventing alopecia (hair loss); and reducing the premature death of skin cells.

The present invention provides pharmaceutical compositions comprising a compound of Formula I in an effective amount to reduce apoptotic cell death in an animal.

The present invention also provides preservation or storage solutions for mammalian organs or tissue, or growth media for mammalian or yeast cells, wherein an effective amount of a compound of Formula I is included in said solutions or media in order to reduce apoptotic cell death in said organs, tissue or cells.

Brief Description of the Figures

Figures 1A-1G depict photographs of HeLa cells challenged with cyclohexamide (CHX) and DMSO (Fig. 1A), tumor necrosis factor-alpha (TNF- α)/CHX and DMSO (Fig. 1B); 50 μ M BOC-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 1C); 50 μ M Cbz-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 1D); 50 μ M Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 1E); 50 μ M Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 1F); and DMSO (Fig. 1G).

Figures 2A-2G depict photographs of HeLa cells challenged with cyclohexamide (CHX) and DMSO (Fig. 2A), TNF- α /CHX and DMSO (Fig. 2B); 5 μ M BOC-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 2C); 5 μ M Cbz-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 2D); 5 μ M Cbz-Glu(OMe)-Val-

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Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 2E); 5 μ M Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 2F); and DMSO (Fig. 2G).

Figures 3A-3G depict photographs of HeLa cells challenged with cyclohexamide (CHX) and DMSO (Fig. 3A), TNF- α /CHX and DMSO (Fig. 3B); 0.5 μ M BOC-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 3C); 0.5 μ M Cbz-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 3D); 0.5 μ M Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 3E); 0.5 μ M Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F, TNF- α /CHX (Fig. 3F); and DMSO (Fig. 3G).

Fig. 4 depicts a bar graph showing the protection of HeLa cells from TNF- α /CHX with various concentrations of Cbz-Val-Asp(OMe)-CH₂F (a) compared to Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (b).

Fig. 5 depicts a bar graph showing the protection of HeLa cells from TNF- α /CHX with various concentrations of Cbz-Val-Asp(OMe)-CH₂F (a) and BOC-Asp(OMe)-CH₂F (b).

Fig. 6 depicts a graph showing the protection of HeLa cells from TNF- α /CHX with various low doses of Cbz-Val-Asp(OMe)-CH₂F (a) compared to Cbz-Val-Ala-Asp(OMe)-CH₂F (b).

Figs. 7A-7E depict the results of PARP cleavage assays in Jurkat cells. Compound 1 = Cbz-Val-Asp(OMe)-CH₂F. Compound 2 = Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F. Compound 3 = Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F. Compound 4 = Cbz-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F. Compound 5 = BOC-Asp(OMe)-CH₂F. Compound 6 = Cbz-Asp- α -([2,6-dichlorobenzoyloxy]methylketone). Compound 7 = Cbz-Val-Ala-Asp(OMe)-CH₂F.

Figs. 8A and 8B depict the photographs of PARP cleavage, showing inhibition of PARP cleavage in antiFas treated Jurkat cells by Z-VD-fmk and Z-VAD-fmk.

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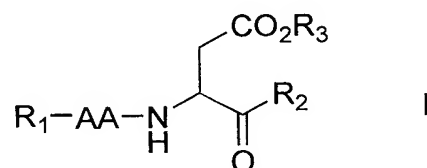
Fig. 9 depicts the graph of cell survival vs Z-VD-fmk concentration, showing inhibition of TNF- α -induced cell death by Z-VD-fmk.

Fig. 10 depicts the photograph of DNA laddering, showing inhibition of DNA laddering in antiFas treated Jurkat cells by Z-VD-fmk.

Figs. 11A and 11B show the neuroprotective effects of systemically administered Cbz-Val-Asp-CH₂F in a rat transient focal ischemia model. Volume of cortical infarction was quantified following 2.25 h of transient focal ischemia and 22 h of reperfusion.

Detailed Description of the Invention

The inhibitors of apoptotic cell death of the present invention are compounds having the general Formula I:



or pharmaceutically acceptable salts or prodrugs thereof, wherein:

R₁ is an N-terminal protecting group including t-butyloxycarbonyl, acetyl, and benzyloxycarbonyl; AA is a residue of any natural α -amino acid, or β -amino acid, e.g. Gly, Thr, Glu, Lys, Arg, Ser, Asn, Gln, Val, Ala, Leu, Ile, Met, and β -amino acids such as β -Ala, and which is not His, Tyr, Pro or Phe; R₂ is H or CH₂R₄, R₄ is an electronegative leaving group such as F, Cl, TsO-, MeO-, ArO-, ArCOO, ArN-, and ArS-; and R₃ is alkyl or H.

With respect to R₃, preferred alkyl groups are C₁₋₆ alkyl groups, e.g. methyl, ethyl, propyl, isopropyl, isobutyl, pentyl and hexyl groups.

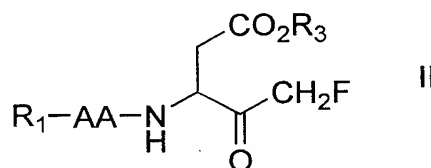
The invention relates to the discovery that the dipeptide-based caspase inhibitors represented by Formula I, though less potent than tri- and

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tetrapeptide inhibitors on the enzymes, are surprisingly potent inhibitors of apoptosis in cell based systems. These compounds are systemically active *in vivo* and are potent inhibitors of antiFas-induced lethality in a mouse liver apoptosis model and have robust neuroprotective effects in a rat model of ischemic stroke. These inhibitors will slow or block cell death in a variety of clinical conditions and industrial applications in which the loss of cells, tissues or entire organs occurs. Therefore, the invention is also related to methods of treating, preventing or reducing conditions in which apoptosis plays a role. These conditions are more fully described below.

The methods comprise administering to an animal in need of such treatment an inhibitor of the present invention, or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit apoptotic cell death.

Preferred embodiments of the compounds that may be employed as inhibitors of apoptosis are represented by Formula II:



or pharmaceutically acceptable salts or prodrugs thereof wherein AA, R₁ and R₃ are as defined previously with respect to Formula I.

Preferred R₁ is t-butyloxycarbonyl, acetyl and benzyloxycarbonyl. Preferred R₃ is H, Me, Et or t-Bu. Preferred AA is Val, Ala, Leu, Ile, Met, and β-amino acids such as β-Ala.

Exemplary preferred inhibitors of apoptosis having Formula I include, without limitation:

Boc-Ala-Asp-CH₂F,

Boc-Val-Asp-CH₂F,

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Boc-Leu-Asp-CH₂F,
Ac-Val-Asp-CH₂F,
Ac-Ile-Asp-CH₂F,
Ac-Met-Asp-CH₂F,
5 Cbz-Val-Asp-CH₂F,
Cbz-β-Ala-Asp-CH₂F,
Cbz-Leu-Asp-CH₂F,
Cbz-Ile-Asp-CH₂F,
Boc-Ala-Asp(OMe)-CH₂F,
10 Boc-Val-Asp(OMe)-CH₂F,
Boc-Leu-Asp(OMe)-CH₂F,
Ac-Val-Asp(OMe)-CH₂F,
Ac-Ile-Asp(OMe)-CH₂F,
Ac-Met-Asp(OMe)-CH₂F,
15 Cbz-Val-Asp(OMe)-CH₂F,
Cbz-β-Ala-Asp(OMe)-CH₂F,
Cbz-Leu-Asp(OMe)-CH₂F, and
Cbz-Ile-Asp(OMe)-CH₂F.

20 Certain of the compounds of the present invention may exist as stereoisomers including optical isomers. The invention includes all stereoisomers and both the racemic mixtures of such stereoisomers as well as the individual enantiomers that may be separated according to methods that are well known to those of ordinary skill in the art.

25 Examples of pharmaceutically acceptable addition salts include inorganic and organic acid addition salts such as hydrochloride, hydrobromide, phosphate, sulphate, citrate, lactate, tartrate, maleate, fumarate, mandelate and oxalate.

Examples of prodrugs include compounds of Formula I-II wherein R₃ is an alkyl group or substituted alkyl group such as CH₂OCH₃. Further, in the

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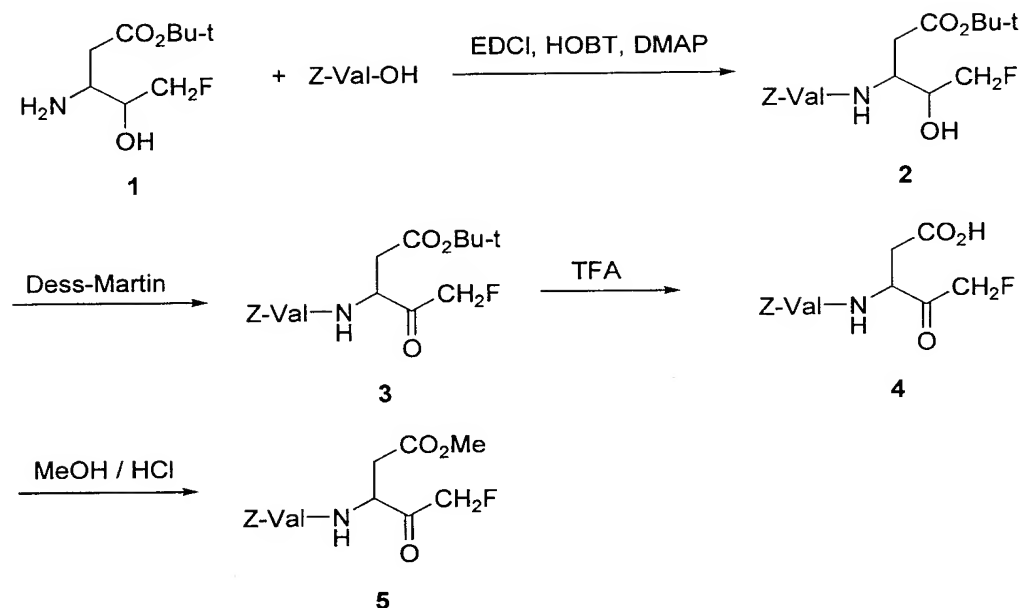
cases where AA contains a carboxylic acid group, examples of prodrugs of formula I-II wherein R_3 is H includes compounds in which either or both carboxyl groups are esterified (e.g. with a C_{1-6} alcohol) or are in the form of the corresponding amides (e.g. with a C_{1-6} amine).

5 The invention is also directed to a method for treating disorders responsive to the inhibition of apoptosis in animals suffering thereof. Particular preferred embodiments of compounds for use in the method of this invention are represented by previously defined Formula II.

10 The compounds of this invention may be prepared using methods known to those skilled in the art. Specifically, compounds with Formulae I-II can be prepared as illustrated by exemplary reactions in Scheme 1. The intermediate **1** was prepared according to Revesz *et al.* (*Tetrahedron Lett.* 35, 9693-9696, 1994). Coupling of **1** with a N-protected amino acid such as Z-Val-OH gave amide **2** which was oxidized by Dess-Martin reagent according to Revesz *et al.* (*Tetrahedron Lett.* 35, 9693-9696, 1994) to give **3**. Acid catalyzed cleavage of the ester gave the free acid **4** which was converted to ester **5**.

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Scheme 1



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An important aspect of the present invention is the discovery that compounds having Formulae I-II are potent inhibitors of apoptosis. Therefore, these inhibitors are expected to slow or block cell death in a variety of clinical conditions in which the loss of cells, tissues or entire organs occurs.

10

The cell death inhibitors of the present invention can be used to reduce or prevent cell death in the nervous system (brain, spinal cord, and peripheral nervous system) under various conditions of ischemia and excitotoxicity, including, but not limited to, focal ischemia due to stroke and global ischemia due to cardiac arrest. One particular usage is to treat the effects of oxygen deprivation which can occur during the birth of infants in high-risk labors.

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The cell death inhibitors can also be used to reduce or prevent cell death in the nervous system due to traumatic injury (such as head trauma), viral infection or radiation-induced nerve cell death (for example, as a side-effect of cancer radiotherapy). The cell death inhibitors can also be used to reduce or prevent cell death in a range of neurodegenerative disorders, including but not limited

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to Alzheimer's disease, Huntington's Disease, Parkinson's Disease, multiple sclerosis, amyotrophic lateral sclerosis, and spinobulbar atrophy. The *in vivo* neuroprotective properties of cell death inhibitors of the invention can be tested in in a rat transient focal ischemia model (Xue *et al.*, *Stroke* 21: 166 (1990)).

The cell death inhibitors of the invention can be used to prevent cell death in any condition which potentially results in the death of cardiac muscle. This includes myocardial infarction, congestive heart failure and cardiomyopathy. One particular application is to reduce or prevent myocardial cell death as occurs in certain viral infections of the heart.

The *in vivo* activity of the cell death inhibitors of the invention can be tested using the "mouse liver apoptosis" model described by Rodriguez *et al.* (Rodriguez *et al.*, *J. Exp. Med.*, 184:2067-2072 (1996)). In this model, mice are treated intravenously (IV) with an antiFas antibody which induces massive apoptosis in the liver and other organs, leading to generalized organ failure and death. This model is useful for indirectly testing the systemic bioavailability of the cell death inhibitors of the invention, as well as their *in vivo* anti-apoptotic properties.

The cell death inhibitors of the invention can be used to prevent cell death of retinal neurons as can occur in disorders which increase intraocular pressure (such as glaucoma) or retinal disorders associated with the aging process (such as age-related macular degeneration). The inhibitors can also be used to treat hereditary degenerative disorders of the retina, such as retinitis pigmentosa.

The cell death inhibitors of the invention can also be used to reduce or prevent premature death of cells of the immune system, and are particularly useful in treating immune deficiency disorders, such as acquired immune deficiency syndrome (AIDS), severe combined immune deficiency syndrome

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(SCIDS) and related diseases. The cell death inhibitors can also be used to treat radiation-induced immune suppression.

Transplantation of human organs and tissues is a common treatment for organ failure. However, during the transplantation process, the donor organ or tissue is at risk for cell death since it is deprived of its normal blood supply prior to being implanted in the host. This ischemic state can be treated with cell death inhibitors by infusion into the donor organ or tissue, or by direct addition of the cell death inhibitors to the organ/tissue storage medium. Cell death inhibitors may also be used to reduce or prevent cell death in the donor organ/tissue after it has been transplanted to protect it from the effects of host immune cells which kill their targets by triggering apoptosis. The cytoprotective effects of cell death inhibitors can also be used to prevent the death of human or animal sperm and eggs used in *in vitro* fertilization procedures. These inhibitors can be used during the harvesting process and can also be included in the storage medium.

Mammalian cell lines and yeast cells are commonly used to produce large amounts of recombinant proteins (such as antibodies, enzymes or hormones) for industrial or medicinal use. The lifespan of some of these cell lines is limited due to growth conditions, the nature of the recombinant molecule being expressed (some are toxic) and other unknown factors. The lifespans of industrial cell lines can be extended by including these cell death inhibitors in the growth medium in a concentration range of 10-200 mM.

The factors governing hair growth and loss are largely unknown. There is some evidence, however, that hair follicle regression (referred to as catagen) may be due at least partially to apoptosis. Therefore, it is contemplated that the cell death inhibitors of the present invention can be used to treat hair loss that occurs due to various conditions, including but not limited to male-pattern baldness, radiation-induced or chemotherapy-induced hair loss, and hair loss due to emotional stress. There is also evidence that

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apoptosis may play a role in the loss of hair color. Therefore, it is contemplated that the cell death inhibitors of the present invention can also be used in treating or preventing cases of premature graying of the hair.

The death of skin epithelial cells can occur after exposure to high levels of radiation, heat or chemicals. It is contemplated that the cell death inhibitors of the present invention can be used to treat, reduce or prevent this type of skin damage. In one particular application, the cell death inhibitors can be applied in an ointment to treat acute over-exposure to the sun and to prevent blistering and peeling of the skin.

Goldberg *et al.* (*Nature Genetics* 13: 442-449 (1996)) reported recently that huntingtin, a protein product of Huntington's disease (HD) gene, can be cleaved by CPP32 but not ICE. The mutation underlying HD is an expansion of a CAG trinucleotide at the 5' end of the HD gene. The trinucleotide expansion exceeding 36 repeats is associated with the clinical presentation of HD. The CAG expansion promotes cleavage of huntingtin by CPP32, thus links the role of CPP32 in the apoptotic cell death in HD. Compounds of the present invention with CPP32 inhibiting activity will be useful in blocking CPP32 induced apoptotic cell death, thus in preventing and treating HD and other disorders characterized by expansion of trinucleotide repeats such as myotonic dystrophy, fragile X mental retardation, spinobulbar muscular atrophy, spinocerebellar atoxia type I and Dentato-Rubro pallidolusian atrophy.

Compositions within the scope of this invention include all compositions wherein the compounds of the present invention are contained in an amount which is effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is with the skill of the art. Typically, the compounds may be administered to mammals, e.g. humans, orally at a dose of 0.0025 to 50 mg/kg, or an equivalent amount of the pharmaceutically acceptable salt thereof, per

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day of the body weight of the mammal being treated for apoptosis-mediated disorders, e.g., neuronal cell death, heart disease, retinal disorders, polycystic kidney disease, and immune system disorders. Preferably, about 0.01 to about 10 mg/kg is orally administered to treat or prevent such disorders. For intramuscular injection, the dose is generally about one-half of the oral dose. For example, for treatment or prevention of neuronal cell death, a suitable intramuscular dose would be about 0.0025 to about 15 mg/kg, and most preferably, from about 0.01 to about 10 mg/kg.

The unit oral dose may comprise from about 0.01 to about 50 mg, preferably about 0.1 to about 10 mg of the compound. The unit dose may be administered one or more times daily as one or more tablets each containing from about 0.1 to about 10, conveniently about 0.25 to 50 mg of the compound or its solvates.

In addition to administering the compound as a raw chemical, the compounds of the invention may be administered as part of a pharmaceutical preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, preferably from about 0.25 to 75 percent of active compound(s), together with the excipient.

Also included within the scope of the present invention are the non-toxic pharmaceutically acceptable salts of the compounds of the present invention. Acid addition salts are formed by mixing a solution of the particular cell death inhibitors of the present invention with a solution of a pharmaceutically acceptable non-toxic acid such as hydrochloric acid, fumaric

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acid, maleic acid, succinic acid, acetic acid, citric acid, tartaric acid, carbonic acid, phosphoric acid, oxalic acid, and the like. Basic salts are formed by mixing a solution of the particular cell death inhibitors of the present invention with a solution of a pharmaceutically acceptable non-toxic base such as sodium hydroxide, potassium hydroxide, choline hydroxide, sodium carbonate and the like.

The pharmaceutical compositions of the invention may be administered to any animal which may experience the beneficial effects of the compounds of the invention. Foremost among such animals are mammals, e.g., humans, although the invention is not intended to be so limited.

The pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, buccal, intrathecal or intracranial routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize

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starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethyl-cellulose phthalate, are used. Dye stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of one or more of the active compounds with a suppository base. Suitable suppository

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bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts and alkaline solutions. Buffers such as Tris may be present. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides or polyethylene glycol-400 (the compounds are soluble in PEG-400). Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

In accordance with one aspect of the present invention, compounds of the invention are employed in topical and parenteral formulations and are used for the treatment of skin damage, such as that caused by exposure to high levels of radiation, including ultraviolet radiation, heat or chemicals.

One or more additional substances which have therapeutic effects on the skin may also be incorporated in the compositions. Thus, the composition may also contain one or more compounds capable of increasing cyclic-AMP levels in the skin. Suitable compounds include adenosine or a nucleic acid hydrolysate in an amount of about 0.1-1% and papaverine, in an amount of about 0.5-5%, both by weight based on the weight of the composition. Also suitable are β -adrenergic agonists such as isoproterenol, in an amount of about 0.1-2% or cyclic-AMP, in an amount of about 0.1-1%, again both by weight based on the weight of the composition. Other suitable types of additional

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active ingredients which may be incorporated in the compositions of this invention include any compounds known to have a beneficial effect on skin. Such compounds include retinoids such as Vitamin A, in an amount of about 0.003-0.3% by weight and chromanols such as Vitamin E or a derivative thereof in an amount of about 0.1-10% by weight, both based on the weight of the composition. Additionally, anti-inflammatory agents and keratoplastic agents may be incorporated in the cosmetic composition. A typical anti-inflammatory agent is a corticosteroid such as hydrocortisone or its acetate in an amount of about 0.25-5% by weight, or a corticosteroid such as dexamethasone in an amount of about 0.025-0.5% by weight, both based on the weight of the composition. A typical keratoplastic agent is coal tar in an amount of about 0.1-20% or anthralin in an amount of about 0.05-2% by weight, both based on the weight of the composition.

The topical compositions of this invention are formulated preferably as oils, creams, lotions, ointments and the like by choice of appropriate carriers. Suitable carriers include vegetable or mineral oils, white petrolatum (white soft paraffin), branched chain fats or oils, animal fats and high molecular weight alcohol (greater than C₁₂). The preferred carriers are those in which the active ingredient is soluble. Emulsifiers, stabilizers, humectants and antioxidants may also be included as well as agents imparting color or fragrance, if desired. Additionally, transdermal penetration enhancers can be employed in these topical formulations. Examples of such enhancers can be found in U.S. Patent Nos. 3,989,816 and 4,444,762.

Creams are preferably formulated from a mixture of mineral oil, self-emulsifying beeswax and water in which mixture the active ingredient, dissolved in a small amount of an oil such as almond oil, is admixed. A typical example of such a cream is one which includes about 40 parts water, about 20 parts beeswax, about 40 parts mineral oil and about 1 part almond oil.

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Ointments may be formulated by mixing a solution of the active ingredient in a vegetable oil such as almond oil with warm soft paraffin and allowing the mixture to cool. A typical example of such an ointment is one which includes about 30% almond oil and about 70% white soft paraffin by weight.

Lotions may be conveniently prepared by dissolving the active ingredient, in a suitable high molecular weight alcohol such as propylene glycol or polyethylene glycol.

In addition, these compositions may include other medicinal agents, growth factors, wound sealants, carriers, etc., that are known or apparent to those skilled in the art. The compositions of the invention are administered to a warm-blooded animal, such as human, already suffering from a skin damage, such as a burn, in an amount sufficient to allow the healing process to proceed more quickly than if the host were not treated. Amounts effective for this use will depend on the severity of the skin damage and the general state of health of the patient being treated. Maintenance dosages over a prolonged period of time may be adjusted as necessary. For veterinary uses, higher levels may be administered as necessary.

In the case of an animal suffering from decreased hair growth, the compositions of the invention are administered in an amount sufficient to increase the rate of hair growth. Amounts effective for this use will depend on the extent of decreased hair growth, and the general state of health of the patient being treated. Maintenance dosages over a prolonged period of time may be adjusted as necessary. For veterinary uses, higher levels may be administered as necessary.

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered

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in clinical therapy and which are obvious to those skilled in the art are within the spirit and scope of the invention.

EXAMPLE 1

t-Butyl 5-fluoro-4-hydroxy-3-nitropentanoate

A solution of oxalic acid (1.9 mL, 21.8 mmol) in dry CH₂Cl₂ (100 mL) was cooled to -78°C, a solution of DMSO (3.0 mL, 42.3 mmol) in dry CH₂Cl₂ (10 mL) was added with stirring in such a rate that the temperature was kept at -50 to -60°C. After 5 min stirring, a solution of 2-fluoroethanol (1.2 mL, 18.4 mmol) in dry CH₂Cl₂ (10 mL) was added, and stirring was continued for an additional 15 min, then dry Et₃N (13.5 mL) was added. The reaction mixture was stirred for 15 min, then allowed to warm to rt. To the reaction mixture was added a solution of t-butyl 3-nitropropionate (2.87g, 16.38 mmol) in CH₂Cl₂ (20 mL). The reaction mixture was stirred at rt for 3 h, then poured into water (100 mL). The organic layer was separated and the aqueous was extracted with CH₂Cl₂ (2 x 50 mL). The CH₂Cl₂ solution was washed with brine, dried and evaporated. The residue was purified by chromatography twice over silica gel (hexane-EtOAc, 7:3) to give 950 mg (24.5%) of the titled product as a colorless viscous oil. ¹H NMR (CDCl₃), 1.450 (s, 9H), 2.80-2.90 (m, 2H), 3.12-3.20 (m, 1H), 4.41-4.59 (m, 2H), 4.57-4.59 (m, 1H), 4.95-5.01 (m, 1H).

EXAMPLE 2

t-Butyl 3-amino-5-fluoro-4-hydroxy-pentanoate

To a solution of t-butyl 5-fluoro-4-hydroxy-3-nitropentanoate (950 mg, 4.0 mmol) in MeOH (20 mL) was added Raney Ni (about 200 mg), the mixture was shaken under H₂ (30-35 psi) at rt for 18 h. It was filtered and the

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catalyst was washed with MeOH (2 x 10 mL). The MeOH solution was evaporated and the residue was purified by chromatography over silica gel (EtOAc-MeOH, 10:1) to give 840 mg (96%) of the titled compound as a yellowish viscous oil. ¹HNMR (CDCl₃), 1.450 (s, 9H), 2.12 (bs, 3H, OH and NH₂), 2.28-2.38 (m, 1H), 2.47-2.57 (m, 1H), 3.24-3.30 (m, 1H), 3.54-3.76 (m, 1H), 4.38-4.48 (m, 1H), 4.54-4.61 (m, 1H).

EXAMPLE 3

t-Butyl 3-(Cbz-Val-amido)-5-fluoro-4-hydroxy-pentanoate

To a solution of Cbz-Valine (396 mg, 1.58 mmol) in THF (20 mL) was added EDCI (300 mg, 1.57 mmol), HOBT (240 mg, 1.57 mmol) and DMAP 129 mg 1.06 mmol). The resulting mixture was stirred for 5 min, then was added a solution of *t*-butyl 3-amino-5-fluoro-4-hydroxy-pentanoate (215 mg, 1.04 mmol) in THF (10 mL) and it was stirred at rt for 18 h. The mixture was filtered and the THF solution was evaporated, and the residue was purified by chromatography over silica gel (hexane-EtOAc, 3:2) to give 290 mg (68%) of the titled compound as a white solid. ¹HNMR (CDCl₃), 0.905 (d, 3H, J=7), 0.965 (d, 3H, J=7), 1.428 (s, 9H), 2.07-2.16 (m, 1H), 2.50-2.57 (m, 1H), 2.64-2.70 (m, 1H), 3.52 (bs, 1H, OH), 3.92-3.96 (m, 2H), 4.20-4.27 (m, 1H), 4.40 (bs, 1H), 4.49 (bs, 1H), 5.10 (s, 2H), 5.31-5.4 (m, 1H, NH), 6.86-6.93 (m, 1H, NH), 7.350(s, 5H).

EXAMPLE 4

Z-Val-Asp-fmk *t*-butyl ester

To a imussion of periodinane (485 mg, 1.14 mmol) in CH₂Cl₂ (20 mL) was added a solution of *t*-butyl 3-(Cbz-Val-amido)-5-fluoro-4-hydroxy-pentanoate (230 mg, 0.52 mmol) in CH₂Cl₂ (12 mL), and the resulting white

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mixture was stirred at rt for 40 min, then poured into 25 mL of saturated aq NaHCO₃ solution containing 1.26 g (8 mmol) of Na₂S₂O₃. The resulting mixture was stirred for 20 min, the resulting clear CH₂Cl₂ solution was separated and the aqueous was extracted with CH₂Cl₂ (2 x 25 mL). The CH₂Cl₂ solution was washed with brine and evaporated, then the residue was purified by chromatography over silica gel (hexane-EtOAc, 3:2) to give 190 mg (83%) of titled compound as a white solid. ¹HNMR (CDCl₃), 0.91-0.97 (m, 6H), 1.415 (s, 9H), 2.10-2.20 (m, 1H), 2.70-2.77 (m, 1H), 2.95-3.01 (m, 1H), 3.98-4.06 (m, 1H), 4.87-5.28 (m, 6H), 6.95-7.01 (m, 1H), 7.350 (s, 5H).

EXAMPLE 5

Z-Val-Asp-fmk

To a solution of the Z-Val-Asp-fmk t-butyl ester (180 mg, 0.41 mmol) in dry CH₂Cl₂ (5 mL) was added F₃CCO₂H (1.0 mL), and it was stirred at rt for 40 min, then evaporated. The residue was purified by chromatography over silica gel (EtOAc-MeOH, 10:1) to give 120 mg (76%) of the titled compound as a white solid. ¹HNMR (DMSO-d₆), 0.81-0.84 (m, 6H), 1.87-1.96 (m, 1H), 2.47-2.67 (m, 2H), 3.77-3.87 (m, 1H), 4.47-4.59 (m, 1H), 4.91-5.16 (m, 4H), 7.25-7.42 (s, 5H), 8.40-8.49 (m, 1H).

The following compounds were obtained by using the same procedure as described in Example 3-5:

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EXAMPLE 6***Z-Leu-Asp-fmk***

5 White solid. ¹H NMR (CDCl₃), 0.87 (m, 6H), 1.11 (m, 1H), 1.47 (m, 1H), 1.81 (m, 1H), 2.7 (m, 1H), 2.95 (m, 1H), 4.10 (m, 1H), 4.80-5.20 (m, 6H), 7.31 (s, 5H).

EXAMPLE 7***Z-Ile-Asp-fmk***

10 White solid. ¹H NMR (CDCl₃), 0.85-0.96 (m, 6H), 1.14-1.26 (m, 1H), 1.422 (s, 9H), 1.87-2.04 (m, 1H), 2.70-2.77 (m, 2H), 2.93-3.00 (m, 1H), 4.02-4.13 (m, 1H), 4.80-5.30 (s, 6H), 6.96 (m, 1H), 7.35 (s, 5H).

EXAMPLE 8***Z-Ala-Asp-fmk***

15 White solid. ¹H NMR (DMSO-d₆), 1.160 (d, 3H, J=7), 2.54-2.70 (m, 2H), 4.00 (m, 1H), 4.54 (m, 1H), 5.10-5.30 (m, 3H), 7.235 (s, 5H), 8.46-8.52 (m, 1H).

EXAMPLE 9***Ac-Val-Asp-fmk***

25 White solid. ¹H NMR (DMSO-d₆), 0.80-0.84 (m, 6H), 1.85-1.97 (m, 4H), 2.56-2.75 (m, 2H), 4.00-4.45 (m, 4H), 5.00-5.30 (m, 2H), 7.85-8.00 (m, 1H), 8.53-8.60 (m, 1H).

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EXAMPLE 10***Z-N-Me-Val-Asp-fmk***

White solid. ¹H NMR (DMSO-d₆), 0.765 (d, 3H, J=7), 0.832 (d, 3H, J=7), 2.06 (m, 1H), 2.57-2.85 (m, 5H), 4.21 (m, 1H), 4.63 (m, 1H), 5.02-5.18 (m, 4H), 7.337 (s, 5H), 8.850 (m, 1H).

EXAMPLE 11***Z-Ala-Asp-fmk***

White solid. ¹H NMR (DMSO-d₆), 2.30 (t, 2H, J=7), 2.48-2.70 (m, 3H), 3.17 (m, 2H), 4.41-4.60 (m, 2H), 4.98-5.30 (m, 3H), 5.40 (m, 1H), 6.63 (m, 1H), 7.32 (s, 5H), 8.52 (m, 1H).

EXAMPLE 12***Z-Gly-Asp-fmk***

White solid. ¹H NMR (DMSO-d₆), 12.50 (s, 1 H), 8.49 (m, 1 H), 7.52 (m, 1 H), 7.33 (s, 5 H), 5.08-5.25 (m, 1 H), 5.01 (s, 2 H), 4.10 (m, 1 H), 3.63 (d, J = 6.0 Hz, 2 H), 2.50-2.80 (m, 2 H).

EXAMPLE 13***Z-Phe-Asp-fmk***

White solid. ¹H NMR (DMSO-d₆), 8.60 (m, 1 H), 7.60 (m, 1 H), 7.24-7.30 (m, 10 H), 4.92 (m, 3 H), 4.60 (m, 1 H), 4.28 (m, 1 H), 2.90 (m, 2 H), 2.70 (m, 2 H).

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EXAMPLE 14***Z-Glu-Asp-fmk***

White solid. ¹H NMR (DMSO-d₆), 12.20 (m, 1 H), 8.48 (m, 1 H), 7.56
5 (m, 1 H), 7.34 (m, 5 H), 5.12 (m, 1 H), 5.01 (s, 2 H), 4.51 (m, 1 H), 3.95 (m, 1
H), 2.71 (m, 2 H), 2.24 (m, 2 H), 1.72-1.82 (m, 2 H).

EXAMPLE 15***Z-Pro-Asp-fmk***

White solid. ¹H NMR (CD₃OD): 7.36-7.33 (m, 5 H), 5.13-5.11 (m, 2
10 H), 4.30 (s, 1 H), 3.58-3.50 (m, 2 H), 2.77-2.64 (m, 2 H), 2.24 (m, 1 H), 1.94
(s, 2 H).

EXAMPLE 16***Z-His-Asp-fmk***

White solid. ¹H NMR (CD₃OD): 8.78 (s, 1 H), 7.93 (s, 1 H), 7.36-7.33
15 (m, 7 H), 5.52 (s, 2 H), 5.10 (s, 2 H), 4.49 (s, 1 H), 3.13-3.05 (m, 2 H), 2.84 (s,
20 2 H).

EXAMPLE 17***Z-Tyr-Asp-fmk***

25 Z-Tyr(Bu-t)-Asp-fmk t-butyl ester was prepared from Z-Tyr(Bu-t)-OH
and t-butyl 3-amino-5-fluoro-4-hydroxypentanoate as described in Examples 3
and 4. To a solution of Z-Tyr(Bu-t)-Asp-fmk t-butyl ester (15 mg, 0.027
mmol) in methylene chloride (1 mL) was added TFA (1 mL). The mixture
was stirred at room temperature for 8 h, then at 4°C for 2 days. It was diluted

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with ethyl acetate (30 mL), washed with water (4 x 20 mL) and brine, dried over Na₂SO₄ and concentrated in vacuo to yield the title compound as a yellow solid (10 mg, 0.022 mmol, 83%). ¹H NMR (DMSO-*d*₆): δ 9.20 (s, 1H), 8.50 (br s, 1H), 7.50 (m, 1H), 7.32-7.03 (m, 7H), 6.63 (d, J = 7.5, 2H), 4.94 (s, 2H), 4.55 (m, 1H), 4.15 (m, 1H), 2.90-2.60 (m, 4H).

EXAMPLE 18

Z-Val-Asp-fmk Methyl Ester

Into a solution of Z-Val-Asp-fmk (110 mg, 0.28 mmol) in MeOH (20 mL) cooled in ice-bath was passed slowly a stream of HCl gas, until the solution turned to strong acidic as determined by pH paper. The solution was stirred at rt for 4 h, then evaporated. The residue was purified by chromatography over silica gel (hexane-EtOAc, 3:2) to give 63 mg (55%) of the titled compound as a white solid. ¹HNMR (CDCl₃), 0.91-0.87 (m, 6H), 2.10-2.20 (m, 1H), 2.81-2.88 (m, 1H), 3.02-3.08 (m, 1H), 3.675 and 3.682 (2S, 3H), 3.97-4.01(m, 1H), 4.90-5.25 (m, 6H), 6.94-7.02 (m, 1H), 7.354 (s, 5H).

The following compounds were obtained by using the same procedure as described in Example 18.

EXAMPLE 19

Z-Leu-Asp-fmk Methyl Ester

Colorless viscous oil. ¹H NMR (CDCl₃), 0.92-0.94 (m, 6H), 1.25-1.80 (m, 4H), 2.78-2.82 (m, 1H), 3.00-3.05 (m, 1H), 3.675 (s, 3H), 4.15-4.20 (m, 1H), 4.85-5.10 (m, 6H), 7.10-7.20 (m, 1H), 7.344 (s, 5H).

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EXAMPLE 20***Z-Ile-Asp-fmk Methyl Ester***

White solid. ¹H NMR (CDCl₃), 0.85-0.96 (m, 6H), 1.14 (m, 1H), 1.46
5 (s, 1H), 1.87 (m, 1H), 1.91-2.86 (m, 1H), 2.88-3.02 (m, 1H), 3.257 (s, 3H),
4.68-4.06 (m, 1H), 4.80-5.30 (s, 6H), 6.99 (m, 1H), 7.35 (s, 5H).

EXAMPLE 21***Cell Death Assays with HeLa Cells***

10 The cytoprotective properties of Cbz-Val-Asp(OMe)CH₂F were tested
using HeLa cells challenged with tumor necrosis factor-alpha (TNF-α) and
cycloheximide (CHX). This is a well-characterized cell culture model of
apoptosis which is commonly used to analyze anti-apoptotic agents. Two
15 types of experiments were performed: a qualitative assessment of cell death by
visualization of the cells using phase contrast microscopy; and a quantitative
assessment of cell death using the fluorescent dye calcein AM.

For photomicroscopy, HeLa cells are seeded in 12-well multidishes at
a density of 100,000 cells per well in Minimal Essential Medium containing 2
20 mM glutamine and 10% fetal bovine serum. 24 hours later the plating
medium is removed and fresh medium is added containing the cytoprotective
test compound at various concentrations. The cells are pre-incubated with test
compound for 2 hours at 37°C in a CO₂ incubator and then TNF-α and CHX
are added at final concentrations of 25 ng/mL and 30 μg/mL, respectively.
25 After a 24 hour incubation period, the cells are examined visually for evidence
of cell death based on cell shape and degree of adherence. Cells are
considered dead if they have become rounded up and phase bright and have
detached from the substratum. Cells are considered alive if they have retained
their normal morphology and remain attached to the substratum.

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For quantitative assays, the degree of cell survival in the presence of test compounds is analyzed quantitatively using the indicator dye calcein AM. This dye is taken up and converted to a fluorescent derivative by living cells; the amount of activated dye in each well can then be assayed in a fluorometric plate reader and the degree of fluorescence is used as a measure of the number of surviving cells. For these assays, HeLa cells are seeded in 48-well plates at a density of 25,000 cells per well in 0.4 mL of Minimal Essential Medium containing 2 mM glutamine and 10% fetal bovine serum. 24 hours later the plating medium is removed and 0.5 mL of fresh medium containing test compound at various concentrations is added. The cells are pre-incubated with test compound for 2 hours at 37°C in a CO₂ incubator and then TNF-α and CHX are added at final concentrations of 25 ng/mL and 30 μg/mL, respectively. After a 24 hour incubation period, the cultures are washed twice with serum-free, phenol red-free Ham's F12 to remove dead cells and 125 μL of Ham's F12 containing 8 μM calcein AM is added. The cultures are incubated at room temperature for 1 hour and the fluorescent signal is determined with a BioTek plate reader using filter settings of 485 nm (excitation) and 530 nm (emission). The data are expressed as "Percent Control," which is calculated by the following equation:

$$\text{Percent Control} = \frac{\text{Calcein AM Signal in the Presence of Test Compound} + \text{TNF-}\alpha \text{ and CHX}}{\text{Calcein AM Signal in the Presence of CHX Only}}$$

The use of CHX-treated cultures as controls, rather than untreated cultures, allows one to correct for the cytostatic effects of CHX. However, because CHX by itself is also a mild inducer of apoptosis in HeLa cells, strong anti-apoptotic drugs will give a Percent Control value greater than 100%.

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Results from a typical qualitative assay are shown in Figures 1A-1G, 2A-2G and 3A-3G. In these experiments the cytoprotective potency of Cbz-Val-Asp(OMe)-CH₂F is compared to the cytoprotective potency of BOC-Asp(OMe)-CH₂F, Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F, and Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F at three different concentrations: 0.5, 5 and 50 μ M. All of these compounds are the methyl ester derivatives. Figures 1A-1G shows that, at a concentration of 50 μ M, Cbz-Val-Asp(OMe)-CH₂F (Fig. 1D) completely protects HeLa cells from the apoptotic effects of TNF- α and CHX. At 50 μ M, the related peptides BOC-Asp(OMe)-CH₂F (Fig. 1C) and Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F (Fig. 1E) are also protective. The CPP32 inhibitor, Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (Fig. 1F), is only marginally effective as a cytoprotective agent at 50 μ M. Figures 2A-2G shows that, at a concentration of 5 μ M, Cbz-Val-Asp(OMe)-CH₂F (Fig. 2D) still shows surprisingly strong cytoprotective action, while 5 μ M BOC-Asp(OMe)-CH₂F (Fig. 2C) and 5 μ M Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F (Fig. 2E) are less effective. The CPP32 inhibitor, Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (Fig. 2F), has no cytoprotective properties at 5 μ M. Figures 3A-3G shows that, even at 0.5 μ M, Cbz-Val-Asp(OMe)-CH₂F (Fig. 3D) is still an effective cytoprotectant, while the other compounds (Figs. 3C, 3E and 3F) give slight or no cytoprotection. These experiments demonstrate that Cbz-Val-Asp(OMe)-CH₂F can protect HeLa cells from TNF- α /CHX-induced apoptosis at concentrations 10 to 100 fold lower than other putative anti-apoptotic agents.

Quantitative experiments using calcein AM, as described above, corroborate the results obtained by microscopic examination. Figures 4 and 5 illustrate the results of two such experiments where the cytoprotective properties of Cbz-Val-Asp(OMe)-CH₂F (a) were compared to BOC-Asp(OMe)-CH₂F (b) (Fig. 5) and Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (c) (Fig. 4) at three concentrations (0.5, 5 and 50 μ M). Fig. 4 shows that

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Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (b) is only minimally effective in protecting HeLa cells from TNF- α /CHX, even at the highest concentration used (50 μ M). Fig. 5 shows that BOC-Asp(OMe)-CH₂F (b) is an effective cytoprotectant at 50 and 5 μ M, but its activity decreases dramatically at 0.5 μ M. By contrast, Cbz-Val-Asp(OMe)-CH₂F (a) is as effective a cytoprotectant at 0.5 μ M as Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (b) is at 50 μ M (Fig. 4). Furthermore, 0.5 μ M Cbz-Val-Asp(OMe)-CH₂F (a) is highly active while 0.5 μ M BOC-Asp(OMe)-CH₂F (b) is inactive (Fig. 5).

In order to determine the effect of Cbz-Val-Asp(OMe)-CH₂F at low doses, HeLa cells were treated with a range of concentrations from 0.05 μ M to 1 μ M. As shown in Fig. 6, Cbz-Val-Asp(OMe)-CH₂F (a) gave significant protection at concentrations as low as 0.25 μ M. By contrast, Cbz-Val-Ala-Asp(OMe)-CH₂F (b), an anti-apoptotic agent widely used in cell death research, shows no cytoprotection in this concentration range.

Taken together, the experiments illustrated by Figures 1A through 6 show that Cbz-Val-Asp(OMe)-CH₂F is a surprisingly potent anti-apoptotic agent in intact cells and is more potent than any other known caspase inhibitor.

EXAMPLE 22

Inhibition of PARP Cleavage in Jurkat Cells

The cleavage of the enzyme poly(ADP)ribose polymerase (PARP) appears to occur in all cells in which the caspase proteolytic cascade is activated. For this reason, PARP cleavage is widely used as a biochemical marker for caspase-mediated apoptosis. The ability of a cytoprotective drug to block PARP cleavage is considered to be an indication of the drug's ability to inhibit the caspase proteolytic cascade and, in particular, CPP32 (caspase-3), the main PARP protease. The ability of Cbz-Val-Asp(OMe)-CH₂F to inhibit

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PARP cleavage was examined during Fas-mediated apoptosis of Jurkat cells, a human T-cell line. This cell culture model of apoptosis is well-characterized and is known to involve activation of at least two caspases, caspase-3 (CPP32) and caspase-8 (FLICE/MACH).

5 For PARP cleavage assays, Jurkat cells were seeded at a density of 500,000 cells per well in six-well multidishes in RPMI 1640 medium containing 10% FBS. The cells were pre-incubated with Cbz-Val-Asp(OMe)-CH₂F or other test compounds for 2 hours at 37°C in a CO₂ incubator and then a monoclonal antibody to Fas was added at a final concentration of 500
10 ng/mL. Incubation at 37°C in a CO₂ incubator was continued for an additional 4 hours. At the end of the incubation period, the cells were harvested by centrifugation and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA and a cocktail of protease inhibitors. An amount of lysate corresponding to 10 to 20
15 µg of protein was loaded on a 7.5% SDS polyacrylamide gel and electrophoresed for 2 to 2.5 hrs at 25mA. The protein was then transferred to a PVDF membrane, probed with a rabbit polyclonal antibody to PARP, and visualized using chemiluminescence.

Figs. 7A-7E shows the result of three such experiments. Jurkat cells
20 were pre-incubated with 0.5, 5, or 50 µM of the following compounds: Cbz-Val-Asp(OMe)-CH₂F (compound 1); BOC-Asp(OMe)-CH₂F (compound 5); Cbz-Asp-α-([2,6-dichlorobenzoyloxy]-methyl ketone) (compound 6), Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F (compound 3), Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F (compound 2), Cbz-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F
25 (compound 4), or Cbz-Val-Ala-Asp(OMe)-CH₂F (compound 7). The cells were then treated with antiFas and processed for Western Blotting. Cbz-Val-Asp(OMe)-CH₂F (compound 1) completely inhibited PARP cleavage at 50 and 5 µM and afforded significant inhibition of cleavage even at 0.5 µM (Fig. 7A). By contrast, Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH₂F

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(compound 2) (Fig. 7A) and Cbz-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F (compound 4) (Fig. 7C) and Cbz-Asp-DCB (compound 6) (Fig. 7D) inhibited PARP cleavage completely at 50 μ M, but were only marginally effective inhibitors at 5 μ M and 0.5 μ M. BOC-Asp(OMe)-CH₂F (compound 5) (Fig. 7D) and Cbz-Val-Ala-Asp(OMe)-CH₂F (compound 7) (Fig. 7E) and Cbz-Glu(OMe)-Val-Asp(OMe)-CH₂F (compound 3) (Fig. 7B) were effective inhibitors of PARP cleavage at concentrations of 50 and 5 μ M, but were only marginally effective at 0.5 μ M, a concentration at which Cbz-Val-Asp(OMe)-CH₂F (compound 1) still showed significant inhibition (Fig. 7A). These experiments demonstrate that Cbz-Val-Asp(OMe)-CH₂F can block the caspase proteolytic cascade in intact cells at a concentration at least 10-fold lower than other known caspase inhibitors.

EXAMPLE 23

Enzyme Activity

The activities of Cbz-Val-Asp(OMe)-CH₂F and Cbz-Val-Asp-CH₂F (free acid) as inhibitors of CPP32, ICE and cathepsin B were measured in a fluorometric enzyme assay. Recombinant CPP32 protein and ICE protein were prepared by expressing DNA clones encoding these enzymes in an insect host cell (sf9 cells) using baculovirus as the vector. See, Webb, N.R. *et al.*, "Expression of proteins using recombinant Baculovirus," *Techniques* 2:173-188 (1990). Preparations of native cathepsin were acquired from a commercial source. Enzyme activity was measured using synthetic peptide substrates attached to a fluorogenic leaving group. Cleavage of the synthetic substrate by the enzyme results in a fluorescent signal which is read in a spectrofluorometer or in a fluorometric microtiter plate reader.

CPP32 activity was measured using the following buffer conditions: 100 mM HEPES pH 7.5, with 10% sucrose, 1% CHAPS, 5 mM glutathione,

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and 5 μ M peptide substrate. The peptide substrate consisted of an oligomer with the sequence Asp-Glu-Val-Asp with the fluorogenic compound aminomethylcoumarin conjugated to the C-terminus. The assay for enzyme activity was typically carried out at 37°C for 30 minutes.

Table I lists the IC₅₀ of Cbz-Val-Asp(OMe)-CH₂F and Cbz-Val-Asp-CH₂F (free acid) for CPP32 and other proteases.

**Table I: Potency of Cbz-Val-Asp(OMe)-CH₂F
and Cbz-Val-Asp-CH₂F (free acid)
as Inhibitors of CPP32 and Other Proteases**

| Enzyme | Cbz-Val-Asp(OMe)-CH ₂ F IC ₅₀ (μ M) | Cbz-Val-AspCH ₂ F (free acid) IC ₅₀ (μ M) |
|--------------------|---|---|
| CPP32 | 1.1 | 0.043 |
| ICE | 0.9 | 0.02 |
| Cathepsin B | 0.3 | >10 |
| Factor Xa | >100 | >100 |
| Thrombin | >100 | >100 |

The results shown in Table I show that compounds of the present invention are moderately potent inhibitors of CPP32 and ICE. It also shows that Cbz-Val-Asp-CH₂F is a potent and selective inhibitor for CPP32 and ICE.

The inhibitory activity of Cbz-Val-Asp-CH₂F in recombinant caspase 3, 6, 7 and 8 obtained from PharMington (a Becton division company, San Diego, CA) were measured using Ac-DEVD-AMC. The amount of each enzyme per assay was as following: 1 ng caspase3, 15 ng caspase6, 2 ng caspase7 and 60 ng caspase8. The enzyme reaction was conducted in 96-well plate using a caspase buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1mM EDTA, 0.1 % CHAPS and 10 % sucrose, pH 7.2) and the reaction was initiated by adding 10 μ M Ac-DEVD-AMC (purchased from Quality

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Controlled Biochemicals, Inc. Hopkinton, MA). Twelve concentrations of Cbz-Val-Asp-CH₂F ranged from 30 pM to 10 μM were tested after incubation of the compound with recombinant caspases for 30 minutes at 37°C. The plate was read with a fluorescence plate reader (EG&G WALLAG, model-1420-002) using excitation filter at 355nm / emission filter at 460 nm. The data was analyzed using GraphPrism software. The data is summarized in table II.

**Table II: Potency of Cbz-Val-Asp-CH₂F
as Inhibitor of caspases**

| | Caspase-3 | Caspase-6 | Caspase-7 | Caspase-8 |
|-----------------------|-----------|-----------|-----------|-----------|
| IC ₅₀ (nM) | 19.8 | 18.4 | 6.8 | 7.2 |

The results shown in Table II show that Cbz-Val-Asp-CH₂F is a potent inhibitor of all the caspases tested.

Table III shows the caspase-3 activity of various dipeptide inhibitors. The results show that Z-Val-Asp-CH₂F is the most potent caspase-3 inhibitor among the compounds tested.

Table III. Caspase-3 activity of the dipeptide inhibitors

| Name | Caspase-3 IC ₅₀ (μM) |
|-----------------|---------------------------------|
| Z-L-Val-Asp-fmk | 0.04 |
| Z-L-Leu-Asp-fmk | 0.2 |
| Z-L-Ile-Asp-fmk | 0.7 |
| Z-L-Phe-Asp-fmk | 0.4 |
| Z-Gly-Asp-fmk | 1.9 |
| Z-L-Ala-Asp-fmk | 0.6 |
| Z-β-Ala-Asp-fmk | 3.5 |

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| Name | Caspase-3 IC ₅₀ (μM) |
|----------------------|---------------------------------|
| Ac-L-Val-Asp-fmk | 0.25 |
| Z-L-Glu-Asp-fmk | 14.2 |
| Z-L-Lys-Asp-fmk.TFA | 1.6 |
| Z-N-Me-L-Val-Asp-fmk | 1.3 |
| Z-L-Pro-Asp-fmk | 0.41 |
| Z-L-His-Asp-fmk | 0.77 |
| Z-L-Tyr-Asp-fmk | 0.66 |

EXAMPLE 24***Effect of Z-VD-fmk on PARP Cleavage***

5 Poly(ADP)ribose polymerase (PARP) was one of the first caspase-3 substrates identified and the cleavage of PARP is still considered to be a near-universal marker for caspase-3 activation and caspase-mediated apoptosis. The ability of an anti-apoptotic compound to block PARP cleavage is therefore a useful indicator of its ability to inhibit apoptosis. The potency of Z-VD-fmk in a PARP cleavage assay was tested using antiFas-treated Jurkat cells. 2 x 10⁶ Jurkat cells were seeded in each well of a 6-well dish and pre-incubated with test compounds for 30 minutes. The cells were then challenged with 500 ng/mL of an agonistic antiFas antibody or PBS for 4 hours. The cells were then harvested, pelleted gently, washed two times with PBS and lysed in RIPA buffer. Aliquots of the lysate were analyzed by SDS-PAGE and the proteins were transferred to PVDF membranes for Western blotting. The primary antibody was a polyclonal antiPARP serum which cross-reacts with both full-length PARP and the caspase-3-generated cleavage product.

20 Fig. 8A shows that Z-VD-fmk completely inhibits PARP cleavage at concentrations of 500 and 250 nM (note absence of the 85 kDa band). Z-VD-

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fmk still retains much of its inhibitory activity even at concentrations as low as 50 nM (Fig. 8A). By contrast, Z-VAD-fmk, although an effective inhibitor of PARP cleavage at 5 μ M (data not shown), is much less effective at 500 nM (Fig. 8B). These experiments show that Z-VD-fmk is at least 10-fold more potent as an inhibitor of PARP cleavage in intact cells than is Z-VAD-fmk and Z-VD-fmk has an IC_{50} value of less than 50 nM in this model of whole-cell apoptosis.

EXAMPLE 25

Effect of Z-VD-fmk on TNF- α -induced Cell Death

Tumor necrosis factor alpha (TNF- α) can trigger apoptosis in a number of cell types by initiating the caspase cascade and its apoptosis inducing activity can be inhibited by peptide-based caspase inhibitors. However, high concentrations of the inhibitors (50 μ M or greater) is required to have good anti-apoptotic effect. HeLa cells, a cell line commonly employed in TNF- α cell death studies, was used here to determine the anti-apoptotic potency of Z-VD-fmk.

HeLa cells were seeded in 48-well multidishes at a density of 50,000 cells per well 24 hours before treatment. They were then pre-incubated with varying concentrations of Z-VD-fmk for 2 hours and challenged with TNF- α (25 ng/mL) and cycloheximide (CHX; 30 μ g/mL). The cultures were incubated for an additional 24 hours and dead cells were removed by two washes with PBS. The density of surviving cells was then measured by incubating each culture for 45 minutes with calcein AM, a profluorescent dye which is taken up by living cells and converted to a fluorescent product. The resulting data were expressed as % control values (control values were cells incubated with cycloheximide but without TNF- α).

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Fig. 9 shows the results of Z-VD-fmk with tested concentrations ranging from zero to 500 nM. Z-VD-fmk provided good cytoprotection at concentrations approaching 100 nM. By contrast, Z-VAD-fmk loses much of its cytoprotective properties below 1 μ M (data not shown). Tetrapeptide inhibitors, such as Z-DEVD-fmk and Ac-DEVD-CHO, are very ineffective below 50 μ M (data not shown). Therefore Z-VD-fmk not only inhibits PARP cleavage at sub-micromolar concentrations (see Example 24), but also inhibits cell death at sub-micromolar concentrations, and is much more effective than known tripeptide and tetrapeptide inhibitors.

EXAMPLE 26

Effect of Z-VD-fmk on DNA Laddering

In the later stages of apoptosis, cells begin to literally fall apart as pieces of the cytoplasm are shed (through blebbing) and the nucleus is disassembled. One of the hallmarks of nuclear disassembly is the cleavage of genomic DNA into nucleosome-sized fragments (termed "DNA laddering"). DNA laddering, like other late stage apoptotic events, is considered to be irreversible, so it is important to determine whether an anti-apoptotic drug can prevent its occurrence.

The ability of Z-VD-fmk to block DNA laddering was tested using antiFas-treated Jurkat cells. Jurkat cells were plated in 60 mm dishes at a density of 5×10^6 cells and pre-incubated with varying concentrations of Z-VD-fmk. They were then challenged with antiFas at 100 ng/mL for 4 hours, harvested, and pelleted and washed with PBS twice. Genomic DNA was isolated using the method of Eldadah et al. (1996). Briefly, the cells were lysed in 2 mL of 7M guanidine HCl and mixed with 1 mL of Wizard miniprep DNA purification resin (Promega). The resin/DNA complex was washed twice with buffer and the DNA was eluted in TE. 1 to 2 μ g of this DNA

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sample was electrophoresed on a 1% agarose/TBE gel and the gel was stained with ethidium bromide.

Fig. 10 shows the results of a DNA laddering assay in which the cells were pre-incubated with Z-VD-fmk or drug vehicle (DMSO). Vehicle treated cells which were challenged with antiFas show a characteristic laddering pattern of DNA extending down to about 300 bp. By contrast, Z-VD-fmk inhibits ladder formation at a dose as low as that of 50 nM.

This result shows that Z-VD-fmk can block a critical late-stage apoptotic event (DNA laddering) at sub-micromolar concentrations comparable to those concentrations which block cell death and PARP cleavage. Based on this experiment and the data described in Examples 24 and 25, it is concluded that Z-VAD-fmk is a highly effective, sub-micromolar apoptotic inhibitor in whole-cell model of apoptosis.

EXAMPLE 27

Anti-apoptotic Activity of Cbz-Val-Asp-CH₂F in the mouse liver apoptosis model

Three to four week old female mice were used in the studies. Liver degeneration was induced by intravenous injection with 2-6 µg of a purified hamster anti-mouse Fas monoclonal antibody (clone Jo2, Pharmingen) against mouse Fas antigen diluted in 80 µl of phosphate buffered physiological saline (Rodriguez, *et al.*, 1996). Mortality was used as the end point to assess liver degeneration. Cbz-Val-Asp-CH₂F was formulated in Tris buffer for intravenous infusion and tested at a dose of 1-10 mg/kg given IV via the tail vein. Ten minutes later, animals were injected with Fas antibody. Mortality was counted at 30 mins, 1, 3 and 24 h. For each compound there were groups of control animals that receive Fas antibody only. Those receiving the highest dose was observed for acute behavioral effects (e.g. sedation, locomoter

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activity, changes in gait, convulsions, straub tail, tremor etc.), and then housed overnight and checked the next day for toxicity / mortality.

In these experiments, Cbz-Val-Asp-CH₂F was a surprisingly potent inhibitor of antiFas-induced lethality. A single 1 mg/kg IV dose completely protected mice from antiFas up to 1 h after antibody administration, and dose as low as 0.25 mg/kg was found to still give almost 100% protection. By contrast, in vehicle control groups all the mice were dead at this time-point. Cbz-Val-Asp-CH₂F also showed substantial protection up to 24 h (28% survival). Separate studies showed that the protection against lethality was associated with the predicted attenuation in the induction of the liver enzymes SPGT and SGOT.

These data demonstrates that Cbz-Val-Asp-CH₂F is highly active *in vivo* following systemic administration in the mouse liver apoptosis model.

EXAMPLE 28

Neuroprotection of Cbz-Val-Asp-CH₂F in Rat Model of Focal Ischemia

(i) *Preparative Surgery:* Male Fischer-344 rats (Harlan Sprague Dawley, CA) weighing 200-240g, were used. Animals were initially anesthetized with 3% halothane in a mixture of 30 % oxygen and 70 % air. Halothane level were reduced to 1.5 % for maintenance of anesthesia throughout surgery. Preparative surgery includes: (a) Intravenous catheter implantation: the left femoral vein were exposed and a Teleflex catheter, filled with vehicle, were inserted up to the inferior vena cava, for subsequent drug administration. (b) Arterial catheter implantation: the femoral artery were cannulated to allow monitoring of the blood pressure, and other physiological conditions include pO₂, pCO₂, pH, glucose, hematocrite, during ischemia, initial drug administration, and the time of arterial reperfusion. Both arterial and venous catheters were exteriorized through the back of the animal, to

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allow free movement. (c) A PhysioTel Transmitter (Data Sciences International, MI) were implanted into the peritoneal cavity to remotely monitor the body temperature of the animals for 22 hours.

(ii) *Physiological parameters*: Core body temperature was maintained at 37.5 °C during surgery, using a YSI Reusable Temperature Probe (YSI Co. Inc., Yellow Spring, OH) connected to a YSI Temperature Control Unit (Model 73A, YSI Co. Inc., Ohio) and a Electric Heating Pad (Model 756, Sunbeam-Oster Co. Inc., Hattiesburg, MS). Following ischemia, the PhysioTel Transmitter was activated and core body temperature was recorded every 5 minutes. Systemic blood pressure was monitored throughout surgery, during intravenous drug infusion (bolus), and 1, 2, 3, and 4 hours after the onset of ischemia. Other physiological conditions, including pO₂, pCO₂, pH, glucose, and hematocrite were examined at the time of arterial occlusion and reperfusion.

(iii) *Transient focal ischemia model*: Following preparative surgery, a ventral midline cervical incision was made to expose both CCAs. The right CCA was permanently ligated with 4-0 silk ligature, while the left CCA was clamped with an atraumatic aneurysm clip. A 1-cm incision perpendicular to and bisecting a line between the lateral canthus of the right eye and the external auditory canal was made. The underlying temporalis muscle was excised and retracted and under direct visualization with the aid of a dissecting microscope (Model SZ-STB1, Olympus, Japan), the middle cerebral artery (MCA) was exposed through a 2 mm burr hole drilled 2-3 mm rostral to the fusion of the zygomatic arch and the squamosal bone. Drilling was done under a continuous irrigation of physiological saline. The dura was cut and retracted to expose the MCA in the rhinal fissure. A Codman micro-aneurysm clip (No.1) was used to temporarily occlude the MCA as it crosses the rhinal fissure. Flow interruption was verified with dissecting microscopic. Incisions were closed with surgical clips, anesthesia was discontinued, and the animals

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were returned to their cages after waking up (within minutes). Rats subject to transient ischemia were reanesthetized after a 2.5 hours after MCA occlusion. After verification of MCA occlusion, the clips on the MCA and left CCA were removed and restoration of blood flow in the MCA confirmed visually. The incision was closed, and rats were returned to their cages. Animals requiring short term recovery were allowed to survive for 24 hours. All animals were deeply anesthetized prior to sacrifice. Brains were removed, and 2 mm coronal sections were sliced and placed in TTC. Infarcted tissue appeared pale, and distinguishable from adjacent viable tissue. The areas of cortical and subcortical infarction were measured blindly with imaging processing software, and the volume of infarction was calculated by adding up individual measures with known thickness.

(iv) *Statistical Analysis:* All physiological parameters, temperature recordings, and the volumes of cortical infarction were compared statistically among experimental and control groups, for each sub-set of animals. Statistical analyses were conducted using Sigmastat software (Jandel Scientific Software, San Rafael, CA). Student's t tests were used for unpaired data and ANOVA for multiple comparisons. A p value of <0.05 was considered significant. Graphs were prepared on SigmaPlot v 2.01 software (Jandel Scientific).

The *in vivo* neuroprotective properties of Cbz-Val-Asp-CH₂F were tested in two stroke studies in the rat (Fischer-344) transient focal ischemia model. Cbz-Val-Asp-CH₂F was given as a 20 mg/kg IV bolus 10 minutes after the onset of ischemia, followed by a continuous IV infusion of 5 mg/kg/hr. In Experiment 1, the continuous infusion was given for 6 h. In Experiment 2, the infusion was extended to 12 h.

Cbz-Val-Asp-CH₂F was found to significantly reduce cortical infarction in both studies: 46% (p <0.05) in Experiment 1 and 57% (p <0.05) in Experiment 2 (Figs. 11A and 11B). There were no changes in blood

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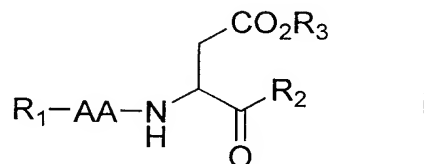
pressure, blood gas, or temperature following drug administration. These results demonstrated that Cbz-Val-Asp-CH₂F is well tolerated following acute IV dosing and is a potent neuroprotectant in a rat model of transient focal cerebral ischemia.

5 Having now fully described this invention, it will be understood by those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their
10 entirety.

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What Is Claimed Is:

1. A compound having the Formula I:



or a pharmaceutically acceptable salt or prodrug thereof, wherein:

R₁ is an N-terminal protecting group; AA is a residue of any natural α-amino acid, or β-amino acid; R₂ is H or CH₂R₄, where R₄ is an electronegative leaving group, and R₃ is alkyl or H; provided that AA is not His, Tyr, Pro or Phe.

2. A compound according to claim 1, wherein R₁ is t-butyloxycarbonyl, acetyl or benzyloxycarbonyl.

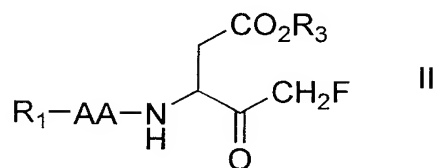
3. A compound according to claim 1, wherein AA is Gly, Thr, Glu, Lys, Arg, Ser, Asn, Gln, Val, Ala, Leu, Ile, Met, or β-Ala.

4. A compound according to claim 1, wherein R₂ is H or CH₂F.

5. A compound according to claim 1, wherein R₃ is H or C₁₋₆ alkyl.

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6. A compound according to claim 1, wherein said compound has the Formula II:



or a pharmaceutically acceptable salt or prodrug thereof, wherein R_1 is an N-terminal protecting group; AA is a residue of any natural α -amino acid, or β -amino acid; and R_3 is alkyl or H; provided that AA is not His, Tyr, Pro or Phe.

7. A compound according to claim 1, wherein said compound is selected from the group consisting of:

Boc-Ala-Asp- CH_2F ,
 Boc-Val-Asp- CH_2F ,
 Boc-Leu-Asp- CH_2F ,
 Ac-Val-Asp- CH_2F ,
 Ac-Ile-Asp- CH_2F ,
 Ac-Met-Asp- CH_2F ,
 Cbz-Val-Asp- CH_2F ,
 Cbz- β -Ala-Asp- CH_2F ,
 Cbz-Leu-Asp- CH_2F ,
 Cbz-Ile-Asp- CH_2F ,
 Boc-Ala-Asp(OMe)- CH_2F ,
 Boc-Val-Asp(OMe)- CH_2F ,
 Boc-Leu-Asp(OMe)- CH_2F ,
 Ac-Val-Asp(OMe)- CH_2F ,
 Ac-Ile-Asp(OMe)- CH_2F ,
 Ac-Met-Asp(OMe)- CH_2F ,

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Cbz-Val-Asp(OMe)-CH₂F,
Cbz-β-Ala-Asp(OMe)-CH₂F,
Cbz-Leu-Asp(OMe)-CH₂F, and
Cbz-Ile-Asp(OMe)-CH₂F.

5

8. A compound according to claim 1, wherein said compound is selected from the group consisting of:

Cbz-Val-Asp-CH₂F, and
Cbz-Val-Asp(OMe)-CH₂F.

10

9. A pharmaceutical composition, comprising a compound of claim 1, and a pharmaceutically acceptable carrier.

15

10. A method of inhibiting cell death or a cell or tissue, comprising contacting said cell or tissue with an effective amount of a compound of claim 1.

20

11. A method of treating or ameliorating cell death in the central and peripheral nervous system, retinal neurons, cardiac muscle or immune system cells of an animal, comprising administering to the animal in need of such treatment or ameliorating an effective amount of a compound of claim 1.

25

12. The method of claim 11, wherein said cell death is in the central or peripheral nervous system, and is due to one of:

(a) a condition of ischemia and excitotoxicity selected from the group consisting of focal ischemia due to stroke and global ischemia due to cardiac arrest;

(b) traumatic injury;

(c) viral infection;

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(d) radiation-induced nerve cell death; or

(e) a neurodegenerative disorder selected from the group consisting of Alzheimer's disease, Parkinson's Disease, a prion disease, multiple sclerosis, amyotrophic lateral sclerosis, and spinobulbar atrophy.

5

13. The method of claim 11, wherein said cell death is in the central or peripheral nervous system, and is due to expansion of trinucleotide repeats of specific genes.

10

14. The method of claim 11, wherein said cell death is due to Huntington's Disease.

15

15. The method of claim 11, wherein said cell death is in cardiac muscle tissue, and is due to myocardial infarction, congestive heart failure, cardiomyopathy or viral infection of the heart.

20

16. The method of claim 11, wherein said cell death is in retinal neurons and is due to increased intraocular pressure, age-related macular degeneration or retinitis pigmentosa.

25

17. The method of claim 11, wherein said cell death is in the immune system, and is due to an immune deficiency disorder selected from the group consisting of acquired immune deficiency syndrome, severe combined immune deficiency syndrome and radiation-induced immune suppression.

18. The method of claim 11, wherein said cell death is due to an autoimmune disorder selected from the group consisting of lupus erythematosus, rheumatoid arthritis and type I diabetes.

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19. The method of claim 11, wherein said cell death is due to type I diabetes.

5 20. A method of treating or preventing polycystic kidney disease or anemia/erythropoiesis in an animal, comprising administering to the animal in need of such treatment or preventing an effective amount of a compound of claim 1.

10 21. A method of protecting a mammalian organ or tissue from cell death due to deprivation of normal blood supply, comprising contacting said organ or tissue with an effective amount of a compound of claim 1.

15 22. The method of claim 21, wherein said organ or tissue is present in a storage medium prior to transplant into a mammal.

23. The method of claim 21, wherein said contacting comprises infusion of said compound into the organ or tissue, or bathing of said organ or tissue in a storage medium which comprises said compound.

20 24. A method of reducing or preventing cell death in a donor organ or tissue after it has been transplanted into a host due to the effects of host immune cells, comprising administering to said host in need thereof an effective amount of a compound of claim 1.

25 25. A method of reducing or preventing the death of mammalian sperm or eggs used in *in vitro* fertilization procedures, comprising contacting said sperm or egg with an effective amount of a compound of claim 1.

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26. A method of extending the lifespan of a mammalian or yeast cell line, comprising contacting said cell line with a compound of claim 1.

27. The method of claim 26, wherein said contacting comprises including said compound in a cell growth medium.

28. A method of treating or ameliorating hair loss or premature graying of the hair in a mammal, comprising contacting the hair or hair follicles of the mammal in need thereof with a compound of claim 1.

29. The method of claim 28, wherein hair loss is treated, and said hair loss is due to male-pattern baldness, radiation, chemotherapy or emotional stress.

30. A method of treating or ameliorating skin damage of a mammal due to exposure to high levels of radiation, heat or chemicals, comprising applying to the skin of the mammal in need thereof with a compound of claim 1.

31. The method of claim 30, wherein said compound is applied as part of an ointment.

32. The method of claim 30, wherein said skin damage is due to acute over-exposure to the sun, and wherein said treating reduces blistering and peeling of the skin.

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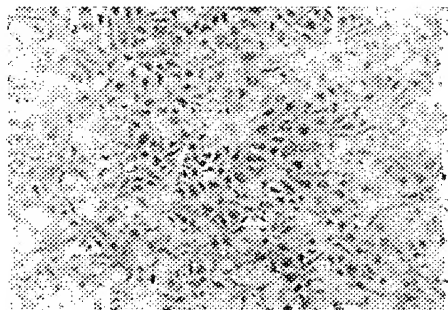


FIG. 1A



FIG. 1B

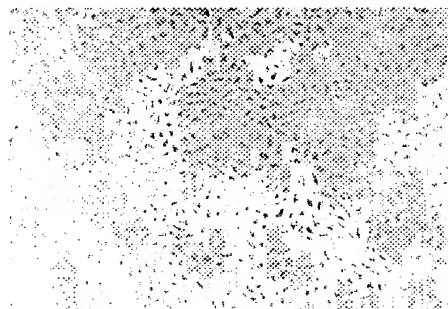


FIG. 1C

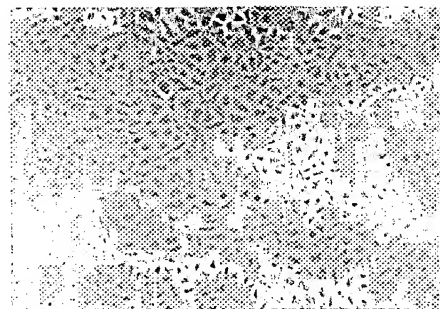


FIG. 1D

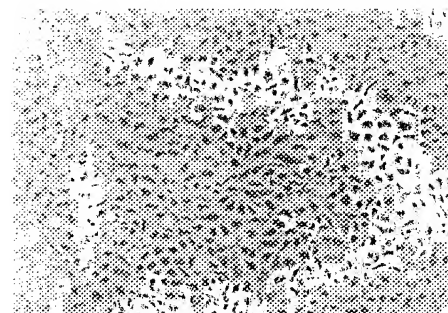


FIG. 1E

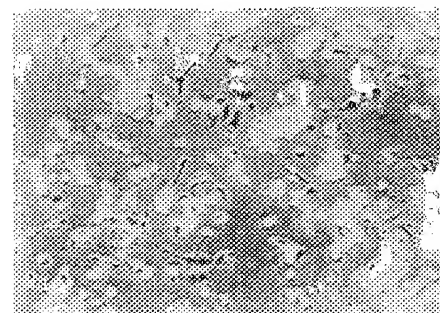


FIG. 1F

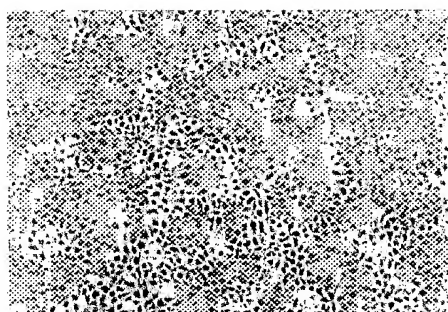


FIG. 1G

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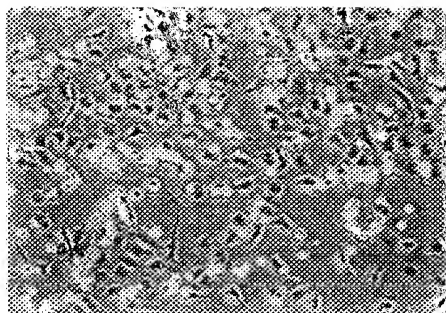


FIG. 2A

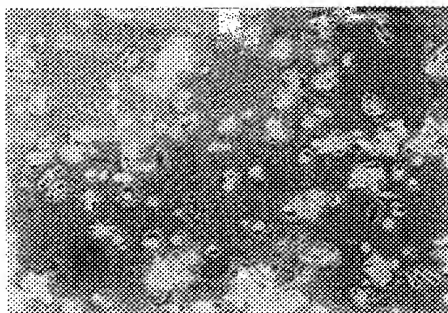


FIG. 2B

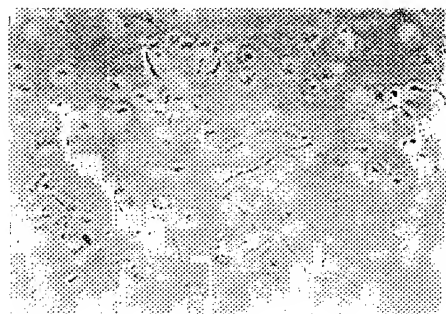


FIG. 2C

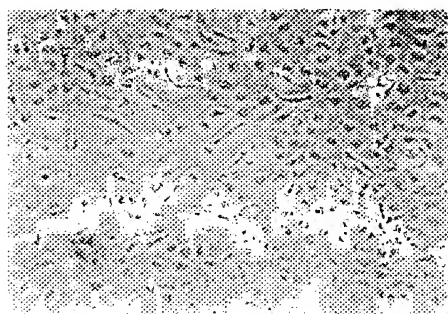


FIG. 2D

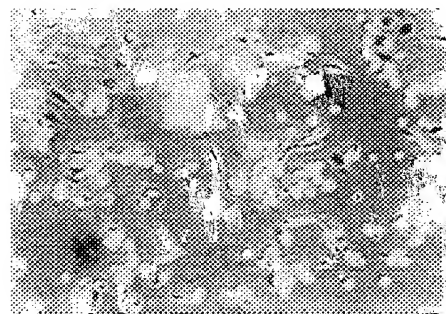


FIG. 2E

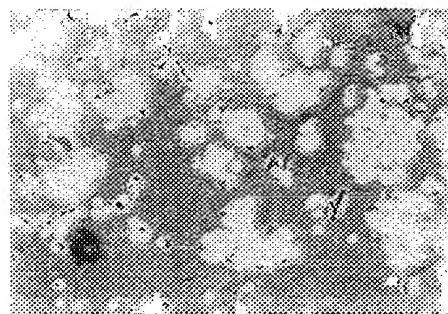


FIG. 2F

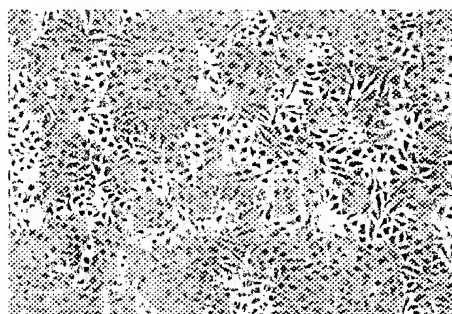


FIG. 2G

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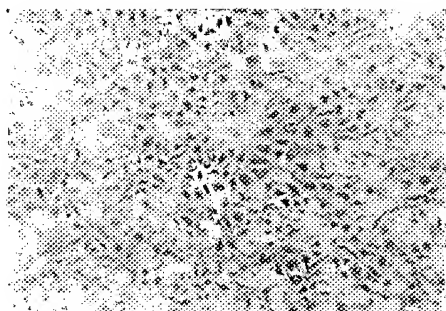


FIG. 3A

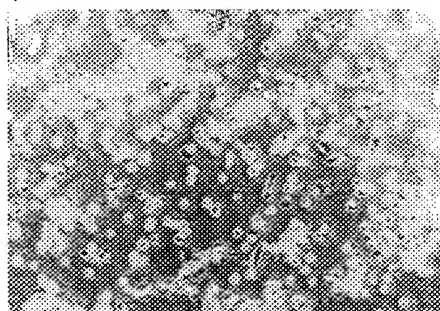


FIG. 3B



FIG. 3C

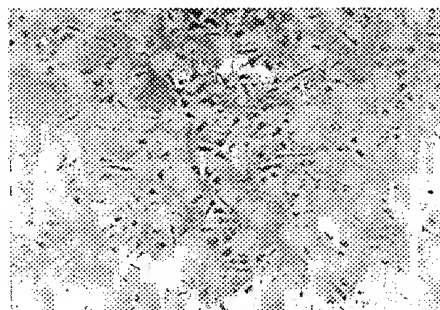


FIG. 3D

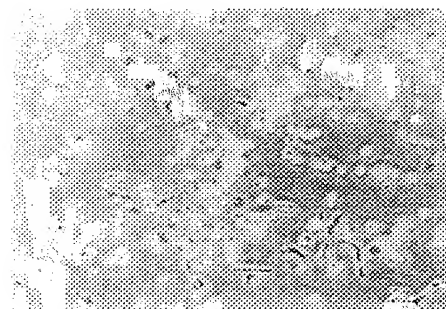


FIG. 3E

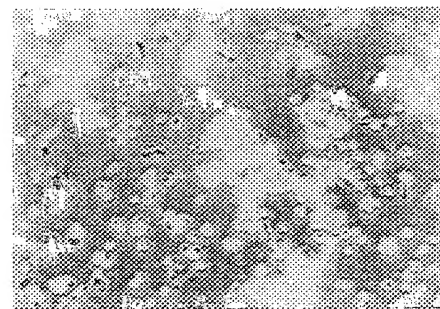


FIG. 3F

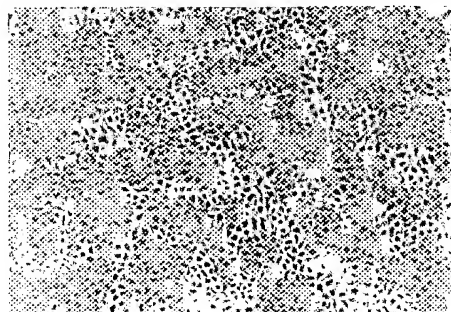


FIG. 3G

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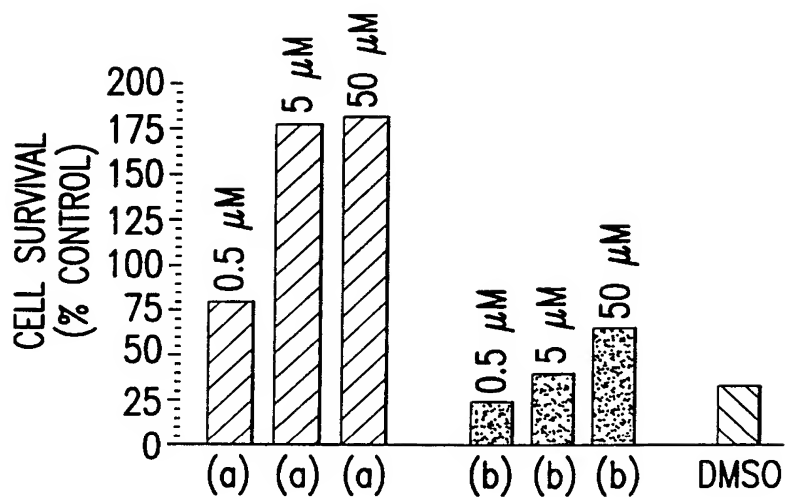


FIG.4

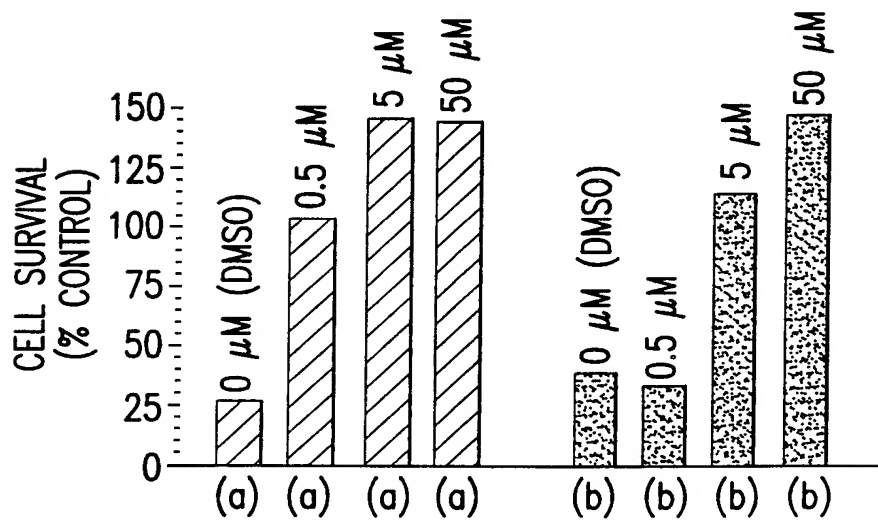


FIG.5

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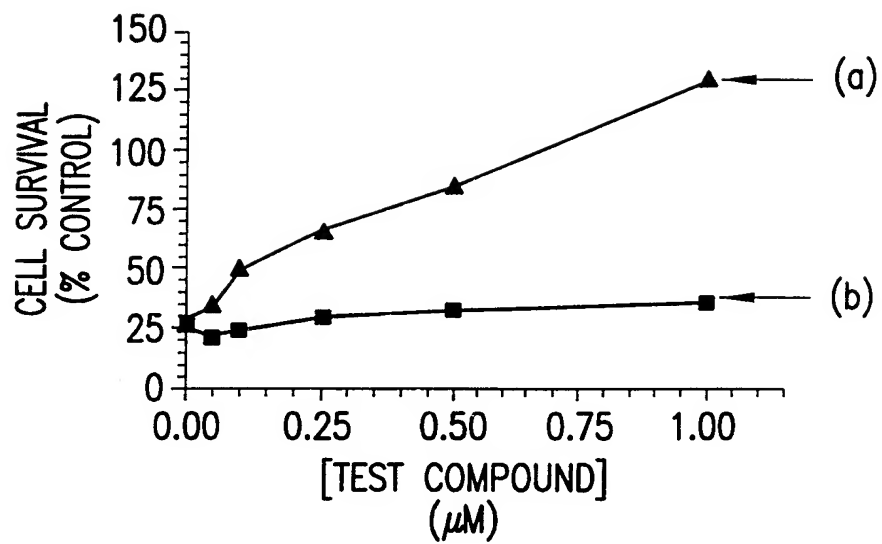


FIG.6

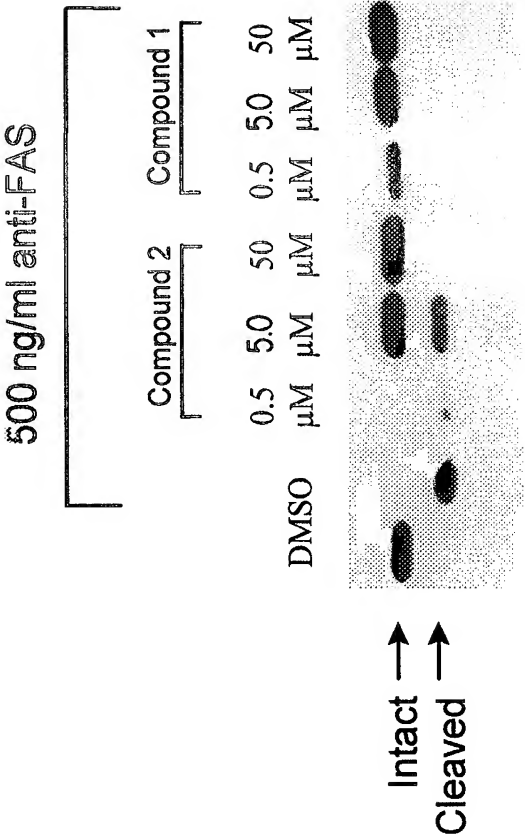


FIG. 7A

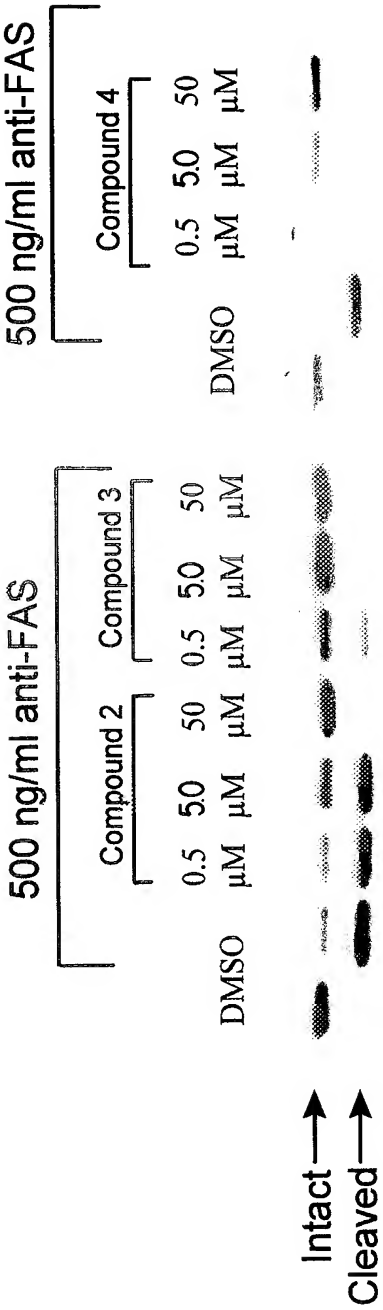


FIG. 7B

FIG. 7C

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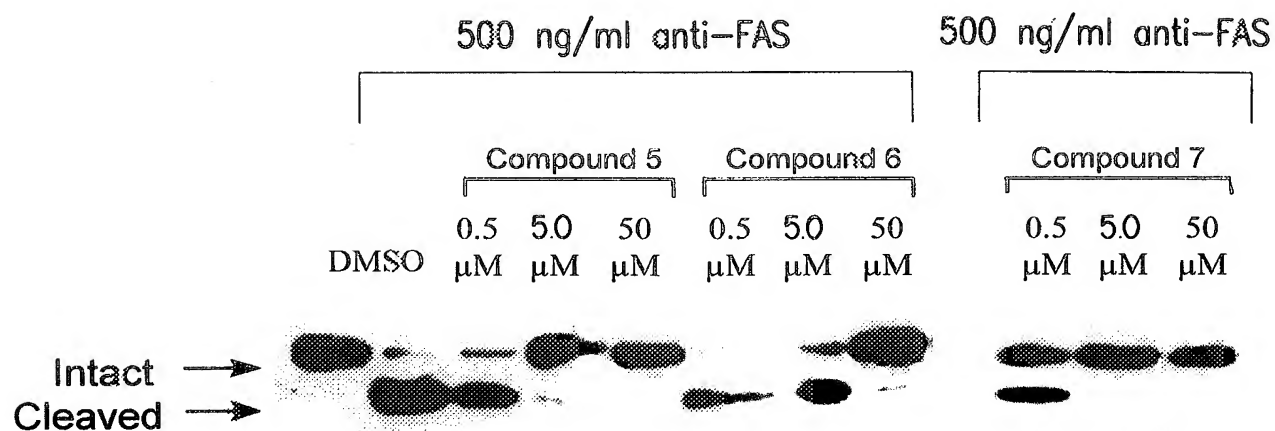


FIG. 7D

FIG. 7E

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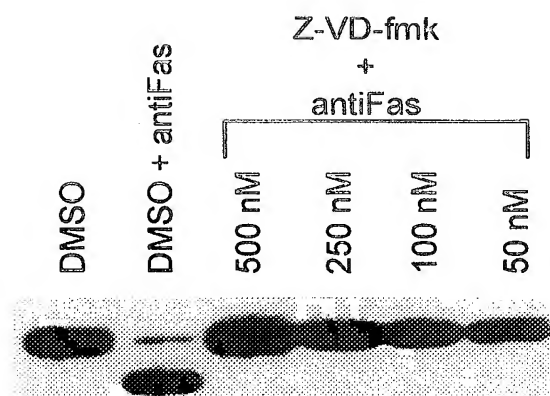


FIG.8A



FIG.8B

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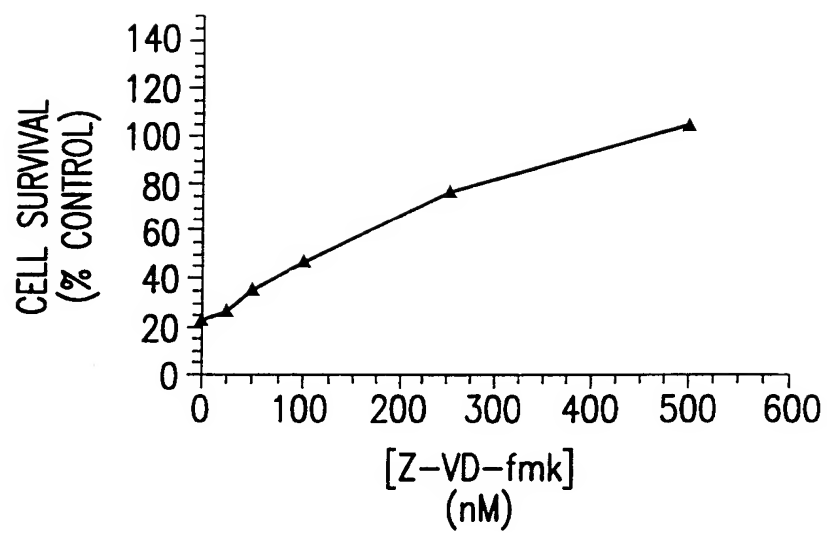


FIG.9

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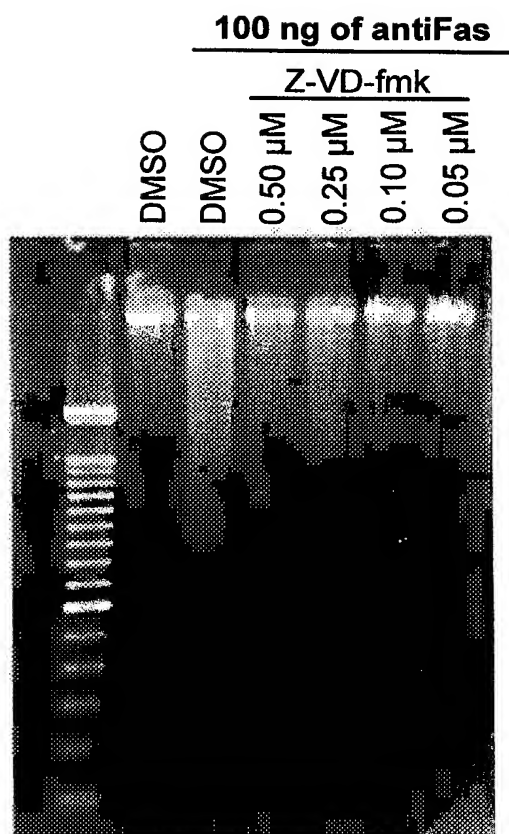


FIG.10

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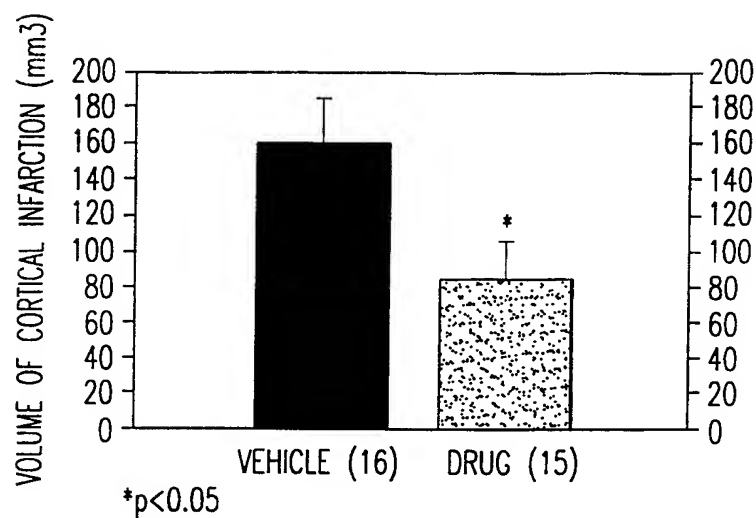


FIG.11A

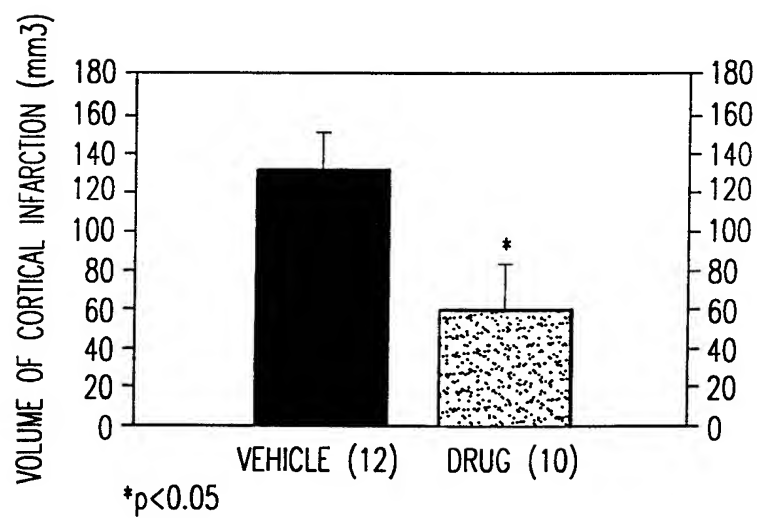


FIG.11B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21232**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 1/02; A61K 38/05; C07K 5/06; C12N 1/04, 5/02

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1.1, 2, 260, 366, 374, 404; 514/19, 20; 548/496; 564/153, 155, 157, 158, 159

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DERWENT, DIALOG, CHEMICAL ABSTRACTS

search terms: ice inhibitor, interleukin inhibitor, caspase inhibitor, aspartic, dipeptide, apoptosis, structure of claim 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | DOLLE et al. P ₁ Aspartate-Based Peptide α -((2,6-Dichlorobenzoyl)oxy)methyl Ketones as Potent Time-Dependent Inhibitors of Interleukin-1 β -Converting Enzyme. Journal of Medicinal Chemistry. 1994, Volume 37, pages 563-564, especially Table 1. | 1, 3, 5, 9-32 |
| X | US 5,434,248 A (CHAPMAN et al.) 18 July 1995, column 2, line 37 - column 6, line 46. | 1-32 |
| X | US 5,462,939 A (DOLLE et al.) 31 October 1995, column 3, lines 31-44, column 8, lines 25-33. | 1-3, 5, 9-32 |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* earlier document published on or after the international filing date | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *G* document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

11 DECEMBER 1998

Date of mailing of the international search report

25 JAN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEFFREY E. RUSSEL

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21232

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X, P | US 5,677,283 A (DOLLE et al.) 14 October 1997, column 6, lines 42-53, column 13, lines 1-27, column 13, line 50 - column 14, line 5, column 14, lines 28-39. | 1-3, 5, 9-32 |
| A | WO 93/05071 A1 (IMMUNEX CORPORATION) 18 March 1993 (18.03.93), page 27, line 34 - page 34, line 21, page 40, line 11 - page 41, line 10. | 1-32 |
| X,P | WO 98/10778 A1 (IDUN PHARMACEUTICALS, INC.) 19 March 1998 (19.03.98), page 9, line 8 - page 11, line 8, page 34, line 1 - page 38, line 17, Figures 1-3. | 1, 3-6, 9-32 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21232

A. CLASSIFICATION OF SUBJECT MATTER:

US CL : 435/1.1, 2, 260, 366, 374, 404; 514/19, 20; 548/496; 564/153, 155, 157, 158, 159